University of Alberta

HORMONAL REGULATION OF δ-AMINOLEVULINATE ACID SYNTHASE IN THE HARDERIAN GLAND OF THE SYRIAN HAMSTER AND OTHER RODENTS

by

KIRSTEN MACMILLAN OATES

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

DEPARTMENT OF ANATOMY AND CELL BIOLOGY

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Fear no more the heat o' the sun,
Nor the furious winter's rages,
Thou thy worldly task hast done,
Home art gone, and ta'en thy wages..
Fear no more the frown o' the great,
Thou art past the tyrant's stroke;
Care no more to clothe, and eat,
To thee the reed is as the oak..
Fear no more the lightning flash,
Nor the all-dreaded thunder-stone,
Fear not slander, censure rash,
Thou hast finished joy and moan..

William Shakespeare

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled HORMONAL REGULATION OF δ-AMINOLEVULINATE ACID SYNTHASE IN THE HARDERIAN GLAND OF THE SYRIAN HAMSTER AND OTHER RODENTS submitted by KIRSTEN MACMILLAN OATES in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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5 June, 1996

This is dedicated to my family, and my dear friends, Leonardo Alvarado, Michael and Thanh Diep, Albert Fabella, and Uri Shanas. All my love for your friendship, encouragement, and support.

ABSTRACT

The hormonal regulation of δ-aminolevulinate acid synthase (ALVS) was investigated in the Harderian gland of the Syrian hamster and other rodents. Western immunoblots were probed with a monoclonal antibody to ALVS and analyzed by densitometry to estimate relative ALVS protein concentrations. Females exhibited higher ALVS protein concentrations than males. ALVS protein amounts were reduced in testosterone-treated females and elevated in castrated males. Exposure to a short photoperiod decreased ALVS protein amounts in intact females, but this decrease was prevented by pinealectomy. In intact and castrated males, low ALVS protein concentrations were maintained, despite the decrease in androgen levels which is induced by short day exposure. Porphyrin levels did not correlate with ALVS protein amounts. Porphyrin was absent, but ALVS protein was present, in Harderian glands from blind mole rats and two species of spiny mice.

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LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
CAS	castrated
ALVS	δ-aminolevulinate acid synthase
EDTA	ethylenediaminetetraacetic acid
INT	intact
LD	long-day photoperiod
MAb	monoclonal antibody
NBT	nitrotetrazolium blue chloride
PMSF	phenyl methyl sulfonyl fluoride
SD	short-day photoperiod
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
TES	testosterone

1 INTRODUCTION

Porphyrins are precursors of heme, which is a prosthetic group of proteins such as hemoglobin, myoglobin, catalase, nitric oxide synthase, peroxidase, cytochromes, and tryptophan pyrrolase (Padmanaban *et al.*, 1989). In heme synthesis, δ–aminolevulinate synthase (ALVS) is the rate-limiting enzyme that catalyzes the conversion of succinate and glycine into δ–aminolevulinic acid. In further reactions, δ–aminolevulinic acid is incorporated into protoporphyrin which is converted into heme by ferrochelatase. Heme feeds back to inhibit ALVS (Padmanaban *et al.*, 1989).

Porphyrins are present in the Harderian gland, which is a large orbital structure. It is interesting that the Harderian glands of Syrian hamsters (*Mesocricetus auratus*) show a marked sexual difference, with female Harderian glands having much higher concentrations of porphyrins than males (Moore *et al.* 1977; Thompson *et al.*, 1984). A reason for the dissimilarity may be that females have either greater porphyrin synthesis or lower porphyrin metabolism than males. The sexual difference is most likely attributed to greater porphyrin synthesis in females as reflected by higher ALVS mRNA and ALVS activity levels (Thompson *et al.*, 1984; Spike *et al.*, 1990; Menendez-Pelaez *et al.*, 1991; Rodriguez *et al.*, 1993).

However, ALVS activity levels are difficult to confirm due to the lability of the enzyme and the lack of techniques sensitive enough to measure accurately its activity in Harderian glands. The goal of my project was therefore to investigate ALVS protein concentrations in Harderian glands. The first specific aim was to determine whether the sexual

dimorphism in porphyrin levels is associated also with greater ALVS protein levels in females compared to males.

Previous studies have suggested that this dimorphism is regulated by androgens, since testosterone treatment of females lowers Harderian porphyrin levels and castration of males increases them (Marrufo *et al.*, 1989; Buzzell *et al.*, 1991). These studies imply that high serum testosterone levels lead to low porphyrin levels, and conversely, that low serum testosterone levels lead to high porphyrin levels. However, it is not clearly understood how testosterone influences porphyrin concentrations. I propose a model in which testosterone decreases porphyrin synthesis. Since the activity of ferrochelatase, the enzyme that metabolizes porphyrin into heme, is very low and identical in female and male hamsters (Thompson *et al.*, 1984), testosterone most likely exerts its effects on porphyrin synthesis. The second aim of my project was to investigate the effects of testosterone on ALVS protein concentrations in female and male hamsters to determine whether testosterone influences porphyrin synthesis.

Hamsters experience seasonal fluctuations in androgen levels, in that long photoperiods (spring and summer) maintain normal androgen levels and short (less than 12.5 hours daily) photoperiods (autumn and winter) decrease them. This drop in androgen levels induces sexual dormancy during the short days of autumn and winter in order to ensure the birth of offspring during a period (spring and summer) that is conducive to their survival (Reiter, 1980).

The effects of short photoperiods are particularly interesting. Exposure to a short photoperiod lowers serum testosterone levels. One would expect therefore that Harderian gland porphyrin levels would

increase. However, Harderian gland porphyrin levels are maintained in hamsters, regardless of seasonal fluctuations in androgen levels (Buzzell *et al.*, 1994). The third aim of my project was to determine the effects of both long and short photoperiods on ALVS protein concentrations to determine whether photoperiod influences porphyrin synthesis.

For the purpose of comparison with the Syrian hamster, the fourth aim was to examine porphyrin and ALVS protein concentrations in other rodents, namely the blind mole rat (*Spalax ehrenbergi*) and two species of spiny mice (*Acomys russatus* and *Acomys cahirinus*).

2 LITERATURE REVIEW

2.1. The Harderian gland

The Harderian gland, a large structure behind the eye, was first identified in 1694 by Johann Jakob Harder in deer as the "glandula nova lachrymalis." It occupies most of the medial orbital cavity. Classification of orbital glands has been based generally upon their location and secretions. In the mammalian eye, the outer canthus is the site of the lateral ocular glands or the lacrimal glands. The inner canthus contains the medial ocular glands, which include the Harderian gland and the nictitans gland (Sakai, 1992). In mammals, these two medial structures are distinguished by their secretions: medial orbital glands which secrete lipids are Harderian glands, whereas those that secrete glycoproteins are nictitans glands.

The medial orbital glands of amphibians, reptiles, and birds are classified as Harderian glands, although their secretions are different from one another and from those of mammals (Chieffi *et al*., 1992; Burns, 1992). The secretions are serous in reptiles, and mucous in birds and amphibians (Payne, 1990).

In contrast, the medial orbital glands of cetaceans, carnivores (cats and dogs), sirenians (dugong), perisodactyls (horse), and some members of the artiodactyls (cow and sheep only), are regarded as nictitans glands because of their histology and histochemistry (Sakai, 1992). Some animals, like the armadillo and hedgehog, possess portions of both Harderian and nictitan tissue in their medial orbital glands (Sakai, 1992).

Despite the complexity in defining a true Harderian gland, its presence or absence has been verified in a variety of animals. It is present in

most terrestrial vertebrates, anuran amphibians, reptiles, birds, and mammals, but is absent in some mammalian carnivores (dogs and cats), all chiropterans (bats), and all primates (Sakai, 1992; Payne, 1994). A rudimentary Harderian gland in humans has been suggested but not verified (Payne, 1994).

The true functions of the Harderian gland have not yet been elucidated, although several theories have been proposed. The secretions of the gland may lubricate the corneal surface of the eye, retard the evaporation of tears, or protect the eye from light (Davis, 1929; Hugo *et al.*, 1987). A growth factor derived from the gland may help to maintain the cornea (Yokoyama *et al.*, 1989). Lymphocytes are particularly prominent in the Harderian glands of birds and may confer immunity to the eye and respiratory tract (Burns, 1992).

A variety of other functions have been suggested. The gland may serve as a source of pheromones. Extensive studies on the Mongolian gerbil indicate that autogrooming releases pheromones from the gland, thereby regulating social behaviors (Thiessen, 1992). In the golden hamster, a homogenate of female gland applied to the snout of a male decreased the frequency of attack behaviors from another male (Payne, 1977). Gland secretions, which are transported from the conjunctival space through ducts into the mouth, may even serve as "saliva" for snakes (Payne, 1994). Lipid-rich Harderian secretions, which are released through the lacrimal canals to the external nares of the nose, are spread by gerbils on their pelage and they may enable them to maintain optimal body temperature and to protect themselves from the cold (Thiessen, 1992). The possible role of the Harderian gland in osmoregulation is still under debate (Payne, 1994).

The Harderian gland is thus present in many species, among which it may have very diverse properties and functions. However, rodents are of major research interest due to the presence of porphyrin within these glands. Rodent Harderian glands are unique in that these glands are major sites for the synthesis and storage of porphyrins. The golden (Syrian) hamster, *Mesocricetus auratus*, has been extensively investigated, because it is unusual among other rodents for the marked sexual differences in many gland features.

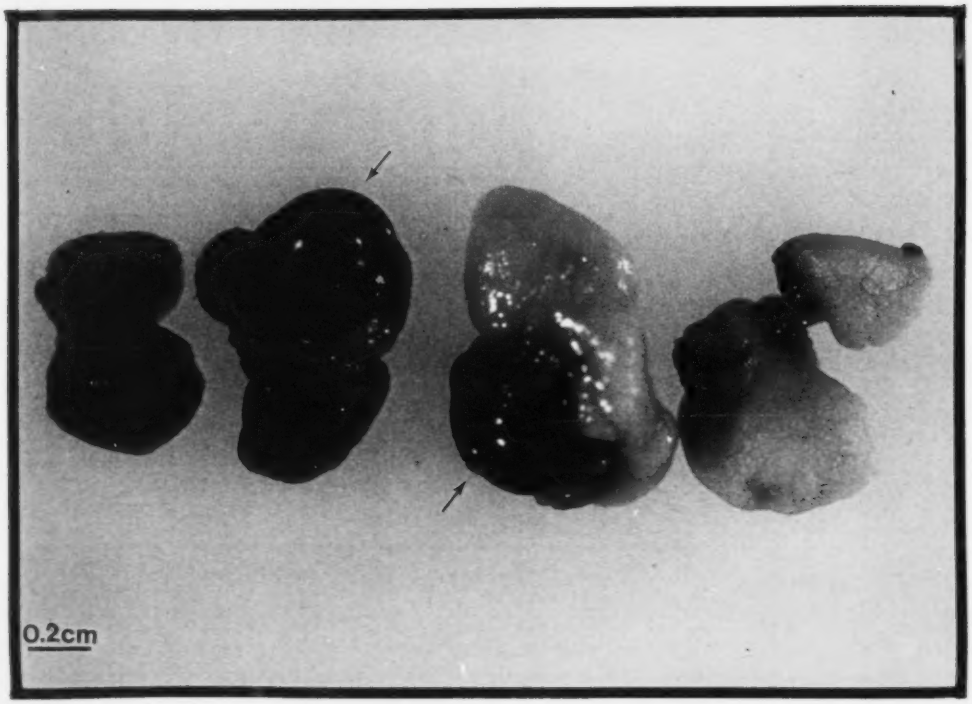
In female and male hamsters, the Harderian gland is conspicuously larger than the eye and occupies most of the orbital cavity (Figure 2.1). Male glands are larger than those of females, and weigh approximately 89 ± 3 mg, whereas those of females weigh about 79 ± 3 mg (Hoffman, 1971). The presence of porphyrin is reflected by the physical appearance of the gland. Female glands are darkly pigmented, reflecting the high porphyrin concentrations, whereas male glands are pale, indicating lower porphyrin levels.

Histological examination reveals that the gland is divided into tubules, with epithelial cells surrounding a central lumen. Luminal contents, epithelial cell types, ultrastructural features, and the quantity of mast cells differ between the sexes. In females, porphyrin accumulates in the lumina as large, precipitated deposits which are absent from the lumina of males (Christensen and Dam, 1953). Female glands contain Type I cells which contain small secretory lipid vacuoles. Male glands include Type II cells which have large lipid droplets, as well as Type I cells (Hoffman, 1971).

An ultrastructural feature, unique in male Harderian epithelial cells, is the polytubular complex which is thought to originate from the smooth

Figure 2.1.

Comparison of female and male Harderian glands. The Harderian glands are horseshoe-shaped, elongate structures that surround the eyeball. The eye (arrow) was included to emphasize the size of the gland in comparison to the eye (B and C). The glands of two females (A and B) and two males (C and D) are shown. Scale bar = 0.2 cm.



A B C D

endoplasmic reticulum (Bucana and Nadakavukaren, 1972). It appears as a series of tubular profiles (both transverse and longitudinal) approximately 30 nm in diameter. Its hypothesized functions include enzyme storage or prevention of porphyrin production and accumulation (Jones and Hoffman, 1976).

Mast cells, located in the interstitial tissue, are forty times more numerous in females than males (Payne *et al.*, 1982). Plasma cells have been observed under certain conditions, namely tubule wall degeneration during aging and ovariectomy (Spike *et al.*, 1986, 1988).

The sexual differences are not confined solely to histological and morphological features. The Harderian glands are biochemically distinct with regard to fatty acids and indoles. In both sexes, about 20% of the wet weight of the gland consists of lipids, principally 1-alkyl-2,3-diacylglycerols. Fatty acids in females include predominantly palmitic acid and branched chains ranging from fourteen to twenty-one carbons, whereas those in males are unbranched and range from twelve to twenty carbons with the majority composed of fifteen carbons (Seyama *et al.*, 1995).

Indoles and the enzymes for their synthesis and metabolism are present in the Harderian gland and differ in the sexes. The major indole is melatonin. The main enzymes include N-acetyltransferase and hydroxyindole-O-methyltransferase which convert serotonin into melatonin, and monamine oxidase which converts serotonin into 5-hydroxyindole acetaldehyde. Hydroxyindole-O-methyltransferase produces 5-methoxyindole acetic acid and 5-methoxytryptophol from this latter product. Female glands have higher levels of melatonin and hydroxyindole-O-methyltransferase but lower levels of N-acetyltransferase than male

glands (Hoffman *et al.*, 1985; Buzzell *et al.*, 1991).

The Harderian glands of the Syrian hamster thus show numerous sexual differences in gross structure, histology, ultrastructure, and biochemistry. Castration of male hamsters converts much (but not all) of the histological, morphological, and biochemical characteristics of male glands to resemble those of female glands. These changes are prevented or reversed by androgen treatment (Hoffman, 1971; Clabough and Norvell, 1973; Payne *et al.*, 1977; McMasters and Hoffman, 1984). Conversely, testosterone treatment of female hamsters generally converts their glandular characteristics to resemble those of males. These findings indicate that several glandular characteristics are under endocrine control. One such characteristic mediated this way is porphyrin.

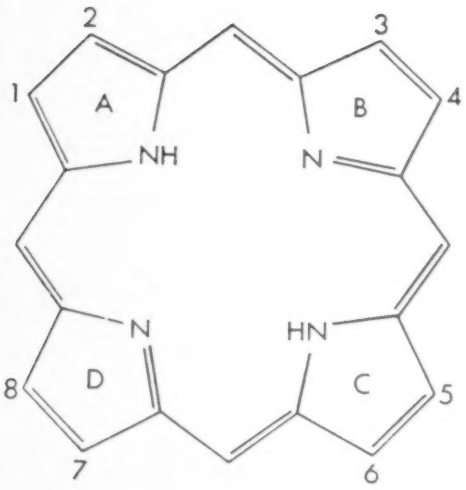
2.2. Porphyrins

Porphyrins are tetrapyrroles, consisting of a ring structure (Figure 2.2). The different types of porphyrins may be distinguished by the methyl, acetyl, propionyl, and vinyl groups attached to specific positions of the structure (Spike *et al.*, 1992). Although they may differ in the constituents that make up their side chains, porphyrins have a basic structure consisting of four pyrrole rings, each with a nitrogen atom (Fessenden and Fessenden, 1986). This arrangement allows porphyrins to complex with different metals to form metalloporphyrins.

An important porphyrin biologically is protoporphyrin, which complexes with iron to form heme. Heme itself is an essential prosthetic group of proteins, such as hemoglobin and myoglobin, and oxidative enzymes, such as catalase, peroxidase, nitric oxide synthase, cytochromes,

Figure 2.2.

Structure of porphyrin indicating four pyrrole rings (A-D) each with a nitrogen atom. Different types of porphyrins are distinguished by the methyl, acetyl, propionyl, and vinyl groups attached to specific positions (1-8). Adapted from Spike *et al*. (1992).


2
3
1
A
B
4
NH
N
N
HN
D
C
8
5
7
6

and tryptophan pyrrolase (Padmanaban *et al.*, 1989). Heme is thus important since it regulates the structure and activity of these proteins and controls many oxygen-requiring systems.

Porphyrins have a variety of characteristics, ranging from their color, distribution, and functions. They are highly colored and fluoresce red when exposed to light with a wavelength near 400 nm (Spike *et al.*, 1992). Porphyrins are key, basic structures that have diverse functions in both plants and animals; for instance, in plants, magnesium bound to modified protoporphyrin forms chlorophyll, a pigment required for photosynthesis. In animals, iron complexed to porphyrin produces heme.

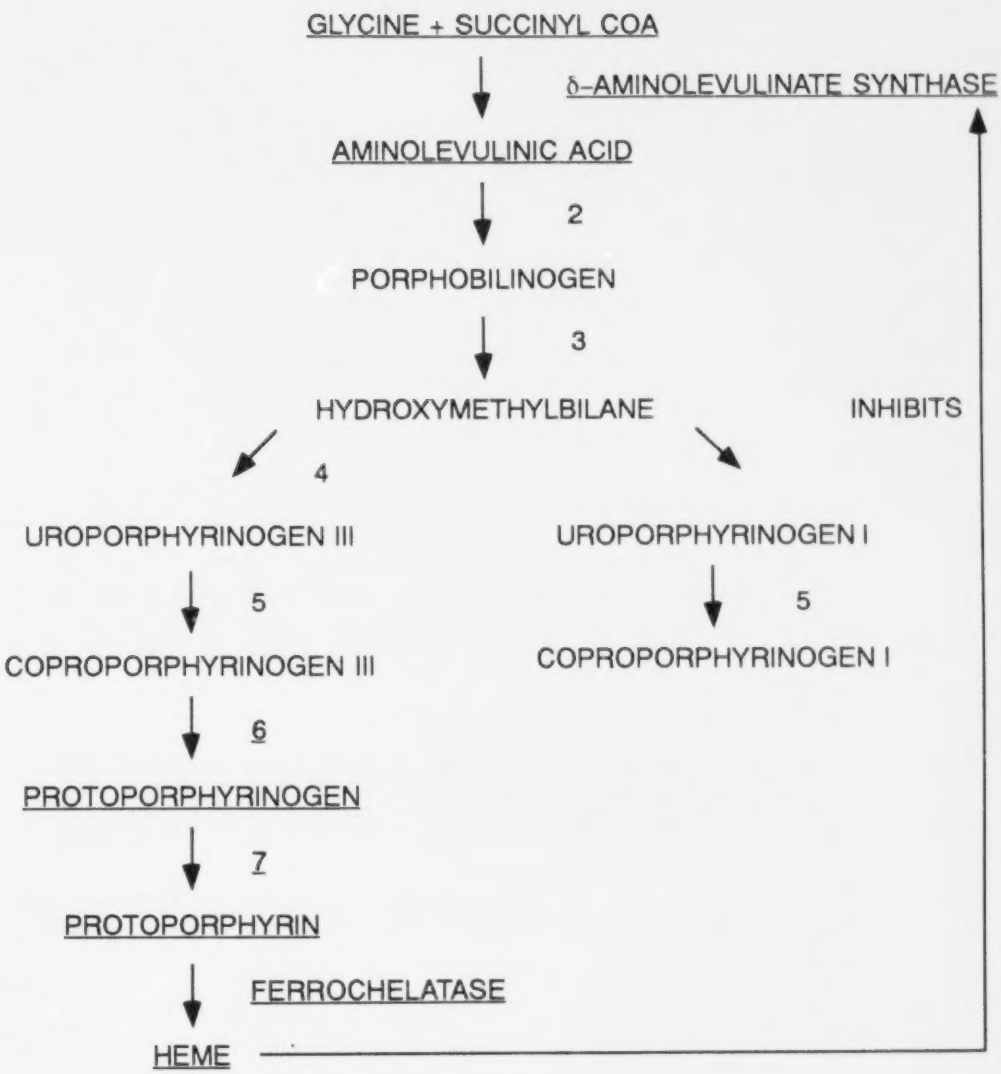
Heme synthesis occurs in most cells, but especially in immature red blood cells and in liver (Padmanaban *et al.*, 1989). Its synthesis involves eight enzymes and a series of irreversible biochemical reactions distributed between the mitochondrion and the cytoplasm (Figure 2.3).

ALVS is the rate-limiting enzyme of the heme synthetic pathway. It is a very unstable enzyme with an *in vivo* half-life of 1 hour in mammals and low endogenous activity (Moore and Disler, 1985). In the mitochondrion, it catalyzes the conversion of succinate and glycine into δ-aminolevulinic acid, which diffuses into the cytoplasm where δ-aminolevulinic acid dehydratase converts it into porphobilinogen (Cartwright, 1977).

The next enzyme, porphobilinogen deaminase, has the second lowest basal activity; hence, it is considered to be the secondary control point (Hindmarsh, 1986). It converts porphobilinogen into hydroxymethylbilane. In the absence of uroporphyrinogen III synthase (the next enzyme), this product will spontaneously form uroporphyrinogen I (Hindmarsh, 1986). In the presence of the enzyme, uroporphyrinogen III is synthesized and the

Figure 2.3.

The pathway for porphyrin and heme synthesis (after Hindmarsh, 1986) indicating the enzymes and reactions occurring in the mitochondrion (underlined text) and in the cytoplasm (plain text). Particular emphasis is given to the initial (δ-aminolevulinic acid synthase) and terminal (ferrochelatase) enzymes of the pathway, and to the negative feedback inhibition of δ-aminolevulinic acid synthase by the end product, heme. Numbers refer to enzymes at those sites: *2*, δ-aminolevulinic acid dehydratase; *3*, porphobilinogen deaminase; *4*, uroporphyrinogen III synthase; *5*, uroporphyrinogen decarboxylase; *6*, coproporphyrinogen oxidase; *7*, protoporphyrinogen oxidase.


GLYCINE + SUCCINYL COA
δ-AMINOLEVULINATE SYNTHASE
AMINOLEVULINIC ACID
2
PORPHOBILINOGEN
3
HYDROXYMETHYLBILANE
INHIBITS
4
UROPORPHYRINOGEN III
UROPORPHYRINOGEN I
5
5
COPROPORPHYRINOGEN III
COPROPORPHYRINOGEN I
6
PROTOPORPHYRINOGEN
7
PROTOPORPHYRIN
FERROCHELATASE
HEME

basic ring structure of a porphyrin is complete. Uroporphyrinogen III is the porphyrin viable for heme biosynthesis due to the fact that it has an asymmetrical structure with the proper substituent orientation (Voet and Voet, 1995).

After uroporphyrinogen decarboxylase has catalyzed its reaction in the cytosol, the synthetic pathway then continues in the mitochondrion. Coproporphyrinogen oxidase and protoporphyrinogen oxidase catalyze subsequent reactions leading to the formation of protoporphyrin. The terminal enzyme, ferrochelatase, then catalyzes the insertion of iron into protoporphyrin to form heme. Once produced, heme is either retained to function within the mitochondrion or transported across the mitochondrial membrane to regulate metabolic processes in the nucleus and cytoplasm (Padmanaban *et al.*, 1989). In the mitochondrion, heme is processed into heme-containing enzymes. In the nucleus, it stimulates transcription of heme-containing proteins, while in the cytoplasm, it regulates the initiation of translation of mRNAs (Padmanaban *et al.*, 1989).

This pathway involves a negative feedback loop. Presumably, the cell maintains a store or "pool" of newly synthesized heme to regulate the pathway (Yamamoto *et al.*, 1982; Hayashi *et al.*, 1983). Heme controls its own synthesis by inhibiting the formation of ALVS. This enzyme is produced in the cytoplasm on free ribosomes as a large precursor protein (Whiting, 1976; Srivastava *et al.*, 1983). After translation, it is then processed into its mature form as it is imported into the inner mitochondrial matrix (Borthwick *et al.*, 1983). Heme blocks the synthesis of ALVS at transcription and translation and prevents the transit of ALVS from the cytoplasm into the mitochondrion (Whiting, 1976; Yamamoto *et al.*, 1982;

Hayashi *et al.*, 1983; Srivastava *et al.*, 1983).

Regulation of the heme synthetic pathway is thus dependent upon the amount of heme that is produced. In this regard, the initial (ALVS) and terminal (ferrochelatase) enzymes of the pathway are significant. If ferrochelatase activity is high, heme will be produced in greater concentrations, inhibit ALVS, and lead to lower levels of δ-aminolevulinic acid and suppressed porphyrin synthesis. Conversely, if ALVS activity is high while ferrochelatase activity is low, more porphyrin than heme will be formed, since minimal heme concentrations are insufficient to inhibit ALVS. As a result, protoporphyrin will accumulate in the tissues without being transformed into heme.

2.3. Porphyrin in the Harderian gland

Among the mammals, rabbits and rodents have porphyrin present in their Harderian glands. All species of rodents have Harderian porphyrin, albeit in differing amounts (Spike *et al.*, 1992). Compared to other tissues of the body, porphyrin levels in the Harderian gland are very high, even exceeding those of the liver and kidney (Payne, 1990). The main type of porphyrin present in Harderian glands is protoporphyrin (the immediate heme precursor), with trace amounts of coproporphyrin and earlier intermediates (Spike *et al.*, 1990).

The function of Harderian porphyrin, however, remains a subject for speculation, since the actual functions of the Harderian gland have not yet been elucidated. Lubrication of the eye and thermoregulation are more likely to be mediated by lipid rather than by porphyrin from the Harderian gland (Spike *et al.*, 1992). Another theory suggests that porphyrin is

converted into heme or hemoproteins, which are required by the gland for some unknown reason.

All enzymes of the heme synthesis pathway are present in the Harderian gland of the Syrian hamster. δ-Aminolevulinic acid dehydratase and ferrochelatase activities are identical in both sexes; however, the activities of the other enzymes (ALVS, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, and protoporphyrinogen oxidase) are higher in females than in males (Formgren and Wetterberg, 1973; Thompson *et al.*, 1984).

With the necessary enzymes present and active in the gland, porphyrin could be synthesized and metabolized into heme or hemoproteins. Since the activity of ferrochelatase (the enzyme that catalyzes the conversion of protoporphyrin into heme) was found to be very low and identical in both sexes (Thompson *et al.*, 1984), considerable heme synthesis is unlikely. Porphyrin synthesis must therefore be predominant in the Harderian gland and in this regard, ALVS, being the rate-limiting enzyme of the pathway, is vital. Higher porphyrin levels in females may be attributed to greater ALVS activity and mRNA levels (Thompson *et al.*, 1984; Spike *et al.*, 1990; Menendez-Pelaez *et al.*, 1991; Rodriguez *et al.*, 1993). However, the levels of ALVS protein, which have not been determined, are likely also significant in regulating porphyrin concentrations, since the level of ALVS activity would depend partly upon the amount of protein present (reflecting the translational rate of ALVS mRNA) and the availability of substrates, which the enzyme and those downstream can use to form porphyrin.

Whereas the regulation of ALVS protein concentrations has not yet been investigated, that of porphyrin concentrations has been extensively studied. Female glands have 100- to 1000- fold higher concentrations of porphyrins than male glands (Moore *et al.* 1977; Thompson *et al.*, 1984). Castration of males (removing testosterone) increases Harderian gland porphyrin concentrations to approach those of females (Buzzell *et al.*, 1991). The effects of castration are reversed by testosterone treatment (Hoffman, 1971; Clabough and Norvell, 1973; Payne *et al.*, 1977; McMasters and Hoffman, 1984). In contrast, testosterone treatment of females diminishes Harderian porphyrin concentrations although not to levels as low as in males and not as dramatic as the rise that is seen in castrated males (Marrufo *et al.*, 1989). These studies suggest that serum testosterone and Harderian porphyrin concentrations are inversely related, with low testosterone leading to high porphyrin concentrations, and high testosterone leading to low porphyrin concentrations.

However, several procedures (blinding, exposure to short photoperiods, and removal of the pituitary gland), which decrease serum testosterone levels, do not produce the effects observed with castration (namely, the rise in porphyrin levels) (Hoffman, 1971; Clabough and Norvell, 1973; McMasters and Hoffman, 1984; Buzzell *et al.*, 1992; Buzzell *et al.*, 1994). Other treatments (administration of bromocriptine, hypothyroidism, and hyperthyroidism) also prevent these effects of castration (Buzzell *et al.*, 1989; Hoffman *et al.*, 1990). These reports suggest that porphyrin levels are not controlled solely by testosterone but by other hormones as well.

Hormonal levels fluctuate naturally in hamsters in accordance with

photoperiod (i.e. short days of winter and long days of summer). Long photoperiods (more than 12.5 hours daily) are required to maintain gonadal size and function, and normal hormonal levels (Reiter, 1973). In contrast, short photoperiods decrease sex steroid, gonadotrophin, prolactin, and thyroid hormone levels, causing reproductive quiescence. In males, atrophy of the testes and accessory sex organs is a consequence of low serum testosterone levels (Reiter, 1967; Reiter, 1968). In females, the ovaries and uterus atrophy and ovulation ceases. Sexual dormancy during winter, followed by gonadal restoration in spring, ensures that offspring are born in spring and summer, increasing their chances of survival.

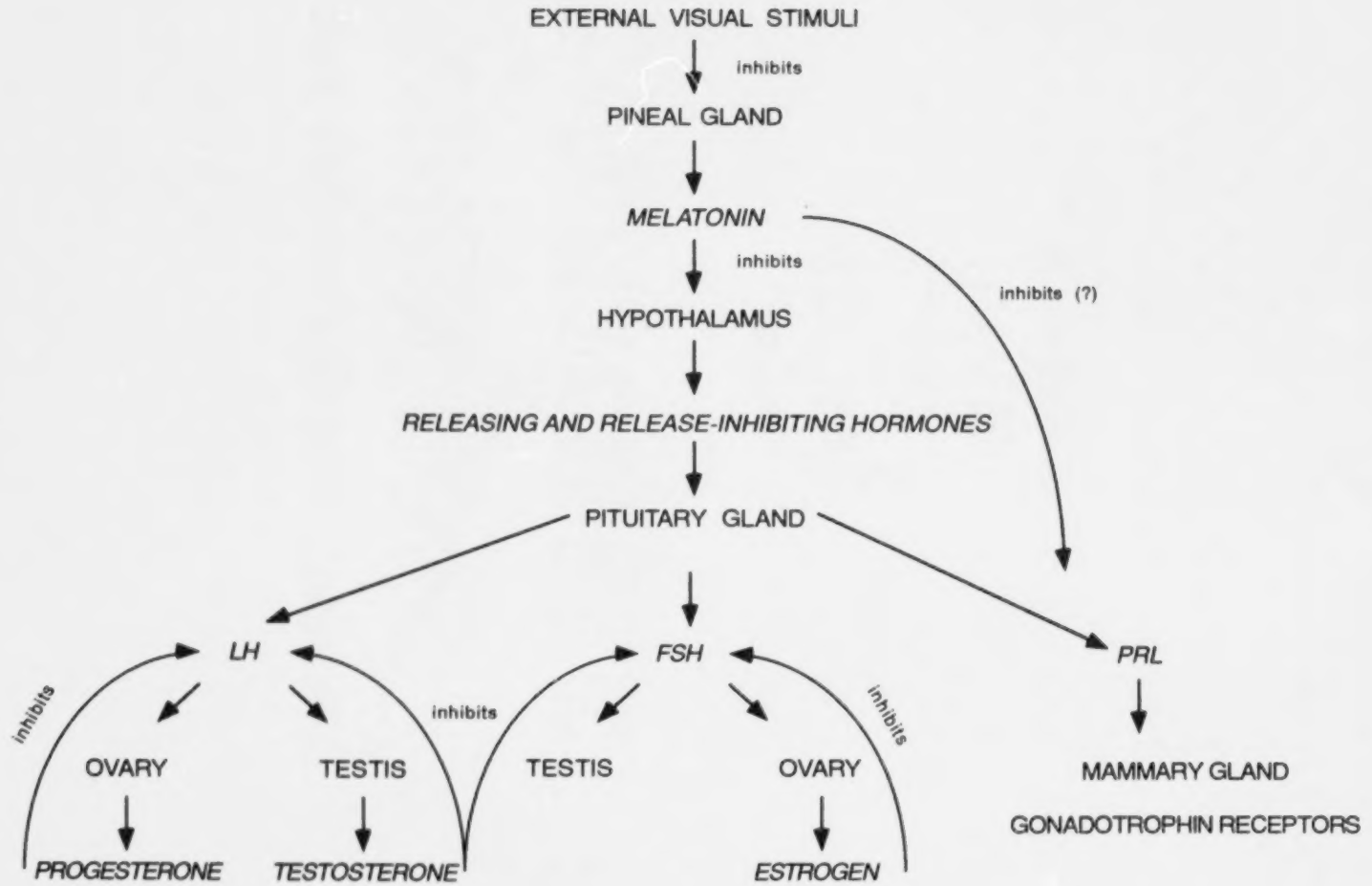
Reproductive quiescence is mediated by the pineal gland, which enables the animal to respond properly to changing photoperiods. Pinealectomy alters the normal reproductive cycles of hamsters, making them sexually functional in all photoperiods (Reiter, 1980).

Stimulated only during darkness, the pineal gland releases melatonin, which affects the production and secretion of other hormones, consequently inhibiting gonadal function (Figure 2.4). Melatonin promotes rhythmic changes in the secretory activity of the neurosecretory cells of the hypothalamus, which in turn influences the production of releasing and release-inhibiting hormones that act on the pituitary gland.

Among the hormones produced by the pituitary gland, prolactin promotes milk secretion from the mammary gland and influences gonadotrophin receptors. Luteinizing hormone stimulates ovulation, which is followed by synthesis of progesterone from the corpus luteum of the ovary in females. In males, luteinizing hormone stimulates the synthesis of testosterone by the Leydig cells of the testes. Follicle-stimulating hormone

Figure 2.4.

Summary of the sources of some hormones and their target organs. Hormones are italicized. Abbreviations: Luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL). Stimulated only in the dark, the pineal gland releases melatonin which inhibits the secretory activity of the hypothalamus, which produces releasing and release-inhibiting hormones that act on the pituitary gland. Pituitary gland hormones include LH, which stimulates the secretion of progesterone from the ovary in females and testosterone from the testes in males; FSH, which stimulates estrogen secretion in females and spermatogenesis in males; and PRL, which promotes milk secretion from the mammary gland and influences gonadotrophin receptors. Progesterone, testosterone, and estrogen regulate their secretion by acting on the hypothalamus or pituitary gland to inhibit the release of LH and FSH.


EXTERNAL VISUAL STIMULI
inhibits
PINEAL GLAND
MELATONIN
inhibits
inhibits (?)
HYPOTHALAMUS
RELEASING AND RELEASE-INHIBITING HORMONES
PITUITARY GLAND
LH
FSH
PRL
inhibits
inhibits
inhibits
OVARY
TESTIS
TESTIS
OVARY
MAMMARY GLAND
GONADOTROPHIN RECEPTORS
PROGESTERONE
TESTOSTERONE
ESTROGEN

induces estrogen secretion by the granulosa cells of the ovary in females and spermatogenesis in males. Progesterone, testosterone, and estrogen regulate their secretion by negative feedback, acting on the hypothalamus or pituitary gland to suppress the release of luteinizing hormone and follicle-stimulating hormone.

In a short photoperiod, melatonin may increase the sensitivity of the hypothalamus, such that even low levels of gonadal hormones inhibit it, preventing the secretion of luteinizing hormone, follicle-stimulating hormone, and prolactin from the pituitary gland, thereby inducing reproductive quiescence (Reiter, 1980).

Besides testosterone, do any of these hormones influence porphyrin concentrations? Melatonin and the enzymes of melatonin synthesis are present in the Harderian gland. However, it is uncertain whether this hormone influences porphyrin levels based upon two contradictory studies (Buzzell *et al.*, 1994; Rodriguez-Colunga *et al.*, 1991). Removing the pituitary gland decreased testosterone, but did not increase porphyrin levels in intact or castrated males, suggesting that some hormone of the pituitary gland is necessary for the effects of castration (i.e. the rise in porphyrin levels) (Buzzell *et al.*, 1992). Progesterone and estrogen do not regulate porphyrin concentrations. Ovariectomized females (lacking progesterone and estrogen) had porphyrin levels similar to those of intact females (Spike *et al.*, 1985; 1986), while progesterone treatment of females and castrated males did not increase porphyrin levels (Menendez-Pelaez *et al.*, 1992).

However, hypophysectomy and replacement of the pituitary gland to the sella turcica (maintaining prolactin levels but low levels of all other pituitary hormones) increased porphyrin concentrations in castrated males,

implying that prolactin regulates porphyrin levels (Buzzell *et al.*, 1992). Studies with bromocriptine supported this observation (Buzzell *et al.*, 1989; Marr *et al.*, 1995). Dopamine from the hypothalamus inhibits prolactin secretion. Bromocriptine is a dopamine agonist and hence reduces prolactin levels. In castrated males, bromocriptine prevented the rise in Harderian gland porphyrin concentrations that follow castration; however, prolactin treatment reversed this effect (Marr *et al.*, 1995). These studies therefore suggest that testosterone and prolactin are involved in the regulation of porphyrin levels in males.

Thyroid hormones also regulate Harderian porphyrin concentrations. Hyperthyroidism (treatment with thyroxine or triiodothyronine) and hypothyroidism (suppression of thyroid function with potassium perchlorate) prevented the rise of porphyrin concentrations in castrated males (Hoffman *et al.*, 1989; Hoffman *et al.*, 1990). In females, hyperthyroidism decreased porphyrin levels, while hypothyroidism had no effect. In addition, treatment with thyroid hormones decreased porphyrin levels in hypophysectomized females and males (Hoffman *et al.*, 1990). Hence, there is evidence that thyroid hormones influence Harderian gland porphyrins.

The sexual difference in porphyrin levels therefore appears to be controlled partly by androgens, although the involvement of pineal, pituitary, and thyroid hormones has been suggested (Hoffman, 1971; Hoffman *et al.*, 1989; Buzzell *et al.*, 1989; Hoffman *et al.*, 1990; Buzzell *et al.*, 1992; Marr *et al.*, 1995). Despite the proposed involvement of other hormones, testosterone undoubtedly is a major regulator of porphyrin levels. However, it is unknown whether testosterone maintains low porphyrin

levels by inhibiting porphyrin synthesis.

Androgen levels, in addition to levels of other hormones, fluctuate in response to photoperiod. Despite a lowering of serum testosterone levels during short photoperiods, porphyrin levels did not increase (Buzzell *et al.*, 1994). The effects of photoperiod on porphyrin synthesis have not previously been examined.

The goal of my project was to test the following hypotheses:

1. That the dimorphism in porphyrin levels between females and males is associated with a difference in ALVS protein levels;
2. That changes in porphyrin levels due to the effects of testosterone and photoperiod are associated with changes in ALVS protein levels.

I studied ALVS protein concentrations by conducting Western blots probed with a MAb for ALVS. The specific aims were:

1. to investigate if the difference in porphyrin levels between female and male hamsters is attributed to a corresponding difference in ALVS protein concentrations between the sexes;
2. to investigate whether testosterone, which affects porphyrin levels, is actually influencing ALVS protein concentrations; and
3. to investigate whether photoperiod, which affects porphyrin levels, is mediating its influence through ALVS protein concentrations.

3 MATERIALS AND METHODS

3.1. Animals

Six-week-old Syrian hamsters (*Mesocricetus auratus*) (Charles River Canada) were housed at 20 ± 2°C, with food and water available *ad libitum*, under controlled photoperiods. Unless otherwise indicated, hamsters were exposed to a photoperiod of 14 hours of light and 10 hours of darkness. The lighting regimen differed in one study (**Chapter 5**) in which animals were kept in short days (8 hours of light per day) to study seasonal phenomena.

Females were monitored for estrous cyclicity for the study in Chapter 5. The 4 day estrous cycle of female hamsters consists of "diestrus day 1", "diestrus day 2," "proestrus," and "estrus," with ovulation occurring around midnight between proestrus and estrus (Orsini, 1961). Females were separated into subgroups based on their time of estrus. The start of their estrous cycle was determined by noting the presence of postovulatory vaginal mucus (Orsini, 1961). Females were sampled at "diestrus day 1" to ensure that the hormonal changes that occur with the estrous cycle did not interfere with the interpretation of the results of the treatments.

3.2. Surgery

Animals were anesthetized with Rompun® (Chemagro Ltd., Etobicote, Ontario) (20 mg/kg) and Ketalean® (M.T.C. Pharmaceuticals, Cambridge, Ontario) (100 mg/kg) by intraperitoneal injection. During surgery, the inhalent anesthetic Metofane® (Pitman-Moore Ltd., Mississauga, Ontario) was administered as necessary to maintain sedation.

Castration of males was via the scrotal route. The skin of the scrotal

sac was cleansed with Betadine. A testis was palpated and an incision was made through the skin of the scrotum and overlying soft tissues of the testis. The testis was then delivered into the wound, a ligature was placed around the spermatic cord, the cord was sectioned and the testis was removed. The skin was then sutured. The procedure was repeated for the other side.

Pinealectomy was performed according to the technique of Hoffman and Reiter (1965). The animal was mounted on a stereotaxic surgical base such that the head was immobilized. A midline sagittal incision was made in the skin between the ears and the subcutaneous soft tissues were scraped from the skull. Using a small disc drill, a disc of bone (5 mm in diameter) overlying the junction of the superior sagittal and transverse sinuses was removed. The pineal gland was removed with fine forceps passed through the junction of the two sinuses. The bone disc was replaced and the wound was closed with surgical clips.

Testosterone pellets were produced by mixing testosterone (Sigma Chemical Co., St. Louis, MO) and beeswax in a ratio of 1:24 (w/w) in a tube. The tube was then placed into a boiling water bath to melt the wax, vortexed and poured onto a creased sheet of aluminum foil. After hardening the wax at room temperature, 25 mg pellets were made and implanted subcutaneously at the nape of the hamster's neck. Fresh pellets were implanted every 2 weeks to maintain the release of testosterone.

3.3. Sampling

All animals were sampled in the morning, unless otherwise indicated. They were anesthetized as described above, weighed, and subsequently decapitated. The weight of each animal and its reproductive organs were

recorded to monitor each animal's physical state and to determine whether the treatments were effective; for example, decreases in the weights of the testes and accessory sex glands are characteristic of short photoperiods. In females, the ovaries and uterus were weighed. In males, the testes and accessory sex glands (seminal vesicles and coagulating glands emptied of their contents) were weighed. The Harderian glands of each animal were dissected, frozen on dry ice, and stored at -70°C until processed for porphyrin determination, SDS-PAGE, or western immunoblotting.

3.4. Determination of Porphyrin Concentrations

Porphyrin concentrations were determined by Dr. G.R. Buzzell as described in Buzzell *et al.* (1989). Tissues were homogenized in an organic phase of ethyl acetate and acetic acid (8:2, v/v), centrifuged, and aliquots of the supernatant added to 1.5 M HCl. Fluorescence was read in a fluorimeter at excitation and emission wavelengths of 405 and 604 nm respectively. Readings were compared with standards prepared from coproporphyrin III tetramethyl ester (Sigma Chemical Co., St Louis, MO) (**Appendix B**).

3.5. Purification of IgG's by Adsorption to Protein A

Monoclonal antibodies to ALVS, present in mouse ascites fluid, were generously provided by Dr. P.G. Bundesen (AGEN Biomedical Ltd., Acacia Ridge, Queensland, Australia). IgG's were purified from ascites fluid using the Affi-Gel Protein A MAPS II Kit (BIORAD, Mississauga, Ontario) according to the manufacturer's instructions. ALVS ascites fluid (500 µL) was diluted to 1:1 with binding buffer and applied to the column. The eluted IgG was dialyzed against phosphate buffered saline (PBS) with 0.05%

sodium azide (pH 7.5). A volume of 7.4 mL, containing 0.13 mg/mL of purified IgG's including ALVS Mab, was collected after dialysis and stored at -70°C until use.

3.6. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting

Tissues were homogenized manually in three volumes of 0.1 M sodium phosphate buffer containing the protease inhibitors, PMSF (0.2 mM) and EDTA (0.5 mM), pH 7.5. PMSF and EDTA were tested on preliminary immunoblots and included to decrease proteolytic products. The homogenate was centrifuged for 2 minutes at 8000 x g. The lipid phase was discarded and the protein concentration of duplicate 2 µL aliquots of the supernatant was measured by the method of Lowry *et al.* (1951) (**Appendix A**).

Samples were prepared in Laemmli (1970) sample buffer and 25 µg of protein were electrophoretically separated at 50 V on 10% polyacrylamide mini gels (0.75 mm thick) with a 3% stacking gel. Proteins from the gels were then transferred to nitrocellulose (S & S NC TM, pore size 0.2 mm, Schleicher and Schuell, Keene, NH) using the BIORAD Mini Trans-Blot® Electrophoretic Transfer Cell at 200 mA for 3 hours.

After transfer, the nitrocellulose was incubated in blocking buffer (5% Carnation® skim milk powder, 50 mM Tris-base, 150 mM NaCl) with 0.3% Tween-20, pH 7.5, overnight at 4°C and then incubated with ALVS MAb diluted 100-fold in blocking buffer with 0.05% Tween-20, pH 7.5, overnight at 4°C.

After 3 washes (10 minutes per wash) in blocking buffer with 0.05%

Tween-20, the blot was incubated with alkaline phosphatase conjugated AffiniPure® Goat Anti-Mouse IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 2000-fold in blocking buffer with 0.05% Tween-20 at room temperature for 2 hours. The blot was developed according to Harlow and Lane (1988), using 50 mg/mL NBT and 50 mg/mL BCIP stock solutions purchased from Fisher/Promega (Madison, WI).

3.7. Densitometry

Immunoblots were analyzed using the BIORAD Model GS-670 Imaging Densitometer and Molecular Analyst® Software (Hercules, CA) on a PowerMac (Cupertino, CA) computer (Model 7100/66). Immunoblots were scanned at a resolution of 64 μm. Equal areas were drawn around the bands of interest and the background of the immunoblot. The optical density was integrated over the area of the band and corrected for background.

4 BACKGROUND STUDIES

Background studies were conducted to validate the techniques used in this thesis. It was essential to determine the specificity of the ALVS MAb and to justify the use of densitometry to indicate the relative quantity of protein present on immunoblots.

4.1. Specificity of the ALVS MAb

4.1.1. Introduction

The ALVS enzyme is synthesized in the cytoplasm on free ribosomes as a large precursor protein (Whiting, 1976; Srivastava *et al.*, 1983), and posttranslationally processed into its mature form as it is imported into the inner mitochondrial matrix (Borthwick *et al.*, 1983). The molecular weights of forms of ALVS protein have varied in published reports, depending upon the method of isolation and purification of ALVS and the animal or tissue of interest (Table 4.1).

The ALVS antibody was prepared in the following steps (Borthwick *et al.*, 1983; Rohde *et al.*, 1990). Chicken embryos were treated with 2-allyl-2-isopropylacetamide and 1,4-dihydro-3,5-dicarbethoxycollidine in order to elevate ALVS protein levels in liver mitochondria. Liver of 18-day-old chicken embryos was homogenized and mitochondria were separated using differential centrifugation. ALVS protein was extracted from the mitochondria using sonication, and ALVS was purified using affinity chromatography for pyridoxal phosphate. BALB/c mice were immunized by injecting ALVS protein subcutaneously. The ascitic (peritoneal) fluid of the

Table 4.1.

Reported Molecular Weight of ALVS

Species	Tissue	Forms of ALVS			Reference
		Precursor (kDa)	Mature (kDa)	Proteolytic Products (kDa)	
Chicken	Liver	-	49	-	Whiting and Granick, 1976
Rat	Liver	75	66	-	Yamamoto *et al.*, 1982
Chicken	Liver	73	65	-	Hayashi *et al.*, 1983
Chicken	Liver	74	68	-	Srivastava *et al.*, 1983
Chicken	Liver	-	65	-	Drew and Ades, 1989
Chicken	Intestine	-	65	-	Drew and Ades, 1989
Chicken	Kidney	-	65	-	Drew and Ades, 1989
Rat	Liver	75	68	45, 33	Rohde *et al.*, 1990

mice was collected. This fluid contained monoclonal antibodies to ALVS.

The molecular weights of forms of ALVS protein have not previously been reported for the Harderian glands of hamsters. The purpose of this experiment was therefore to identify the immunoreactive bands that were recognized by the ALVS MAb in hamster Harderian gland and liver, and to use the liver of a non-drug-treated chicken embryo (at 18-days-old) as a positive control for the specificity of the MAb.

4.1.2. Methods

Tissues of several treated and untreated animals were dissected and processed for SDS-PAGE and western immunoblotting with ALVS MAb (**Section 3.6.**). The tissues included:

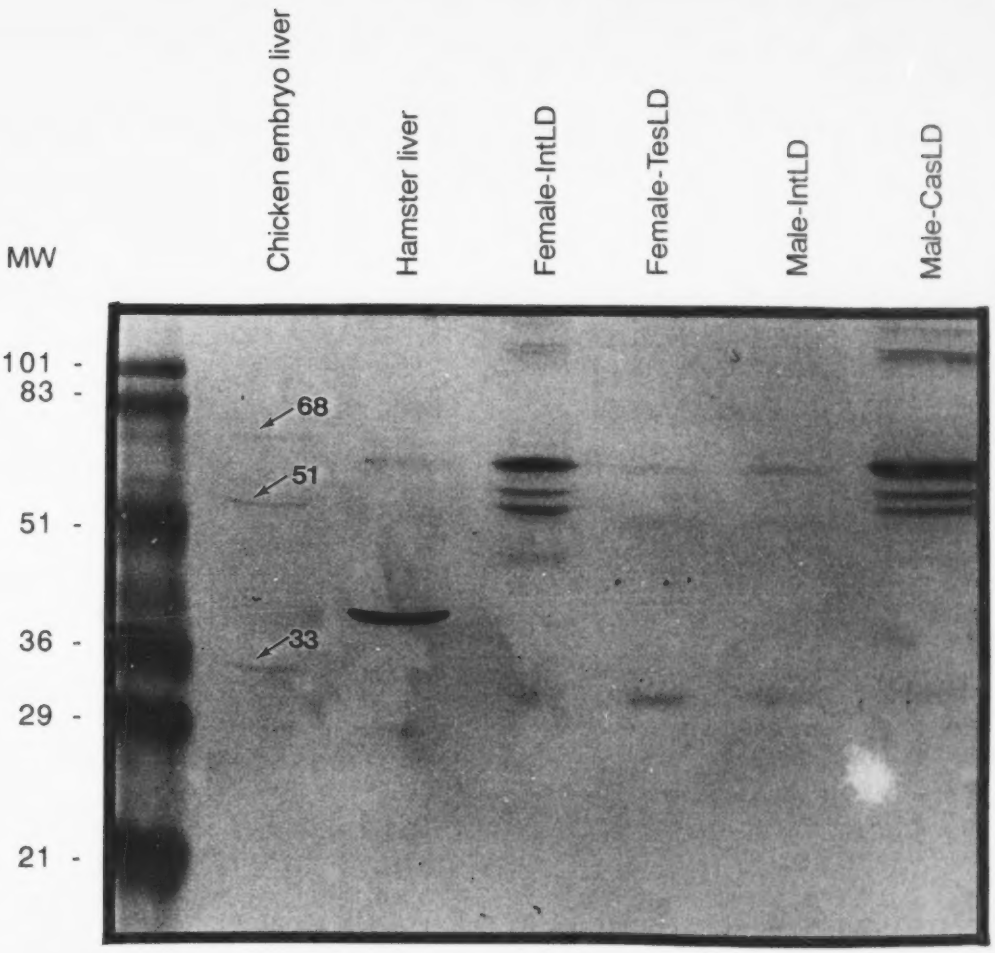
a) The Harderian glands of the following hamsters exposed to a long photoperiod:
   i) one intact female;
   ii) one testosterone-treated female (nine weeks of testosterone treatment);
   iii) one intact male;
   iv) one castrated male (eight and a half weeks postcastration);
b) the liver of a female hamster;
c) the liver of an 18-day-old chicken embryo (non-drug-treated).

4.1.3. Results

Figure 4.1 shows an immunoblot of the liver of a non-drug-treated chicken embryo, female hamster liver, female Harderian gland, and male Harderian gland. Immunoblotting with ALVS MAb produced very faint

Figure 4.1.

Immunoblot analysis of ALVS protein forms in chicken embryo liver, hamster liver, and Harderian glands of intact (Int) and testosterone-treated (Tes) female hamsters, and intact (Int) and castrated (Cas) male hamsters exposed to a long photoperiod (LD). Chicken embryo liver showed very faint bands at 68, 51, and 33 kDa, while hamster liver had bands at 63 and 40 kDa. Bands greater than 101 kDa, and 63, 54, 51, and 44 kDa bands were present in intact female Harderian gland, while 63, 51, and 29 kDa bands were present in testosterone-treated female Harderian gland. Intact male Harderian gland had bands at 63 and 29 kDa, while castrated male Harderian gland had bands greater than 101 kDa, and 63, 54, and 51 kDa. Molecular weight standards (MW): phosphorylase B (101 kDa), bovine serum albumin (83 kDa), ovalbumin (51 kDa), carbonic anhydrase (36 kDa), soybean trypsin inhibitor (29 kDa), and lysozyme (21 kDa).


MW
Chicken embryo liver
Hamster liver
Female-IntLD
Female-TesLD
Male-IntLD
Male-CasLD
101 -
83 -
51 -
36 -
29 -
21 -
68
51
33

bands at 68, 51, and 33 kDa in chicken embryo liver and bands at 63 and 40 kDa in female hamster liver. Bands greater than 101 kDa, and 63, 54, 51, and 44 kDa bands were present in the intact female Harderian gland, while 63, 51, and 29 kDa bands were present, but faint, in the Harderian gland of the testosterone-treated female. A similar situation was observed with the male Harderian glands. Bands at 63 and 29 kDa were present, although faint, in the intact male Harderian gland, but bands greater than 101 kDa and 63, 54, and 51 kDa bands were obvious in the Harderian gland of the castrated male. All bands were darker in the Harderian gland of the castrated male than the intact female.

4.1.4. Discussion

The purpose of this experiment was to confirm that the ALVS MAb, which recognizes ALVS protein in chicken embryo liver, also specifies the same band for ALVS protein in hamster tissue. The ALVS MAb was originally prepared from chicken embryos that had been treated with drugs to elevate ALVS protein levels in the liver. The drugs were not available for this experiment; therefore, the liver of a non-drug-treated chicken embryo was tested, resulting in very faint bands. A 68 kDa band, which represents the mature form of ALVS, was found in chicken embryo liver, corresponding with previous reports of 65-68 kDa (Hayashi *et al.*, 1983; Srivastava *et al.*, 1983; Drew and Ades, 1989). This result confirms that the ALVS MAb specifies ALVS protein.

The ALVS MAb recognized a faint band at 63 kDa and a strong band at 40 kDa in hamster liver. The 63 kDa band is likely the mature form of the enzyme, with the stronger 40 kDa band being a proteolytic product.

Both female and male Harderian glands showed a 63 kDa band representing mature ALVS protein. In addition, the density of the 63 kDa band was stronger in intact female Harderian gland than in intact male Harderian gland. These results indicate a difference in ALVS protein levels between female and male Harderian glands.

The density of the 63 kDa band was weaker in Harderian glands from testosterone-treated females than in intact females. Similarly, this band was weaker in Harderian glands from intact males compared to castrated males. These findings imply that the difference in ALVS protein levels between female and male Harderian glands is mediated by testosterone. This possibility was further explored in other experiments (**Chapter 5**).

Bands greater than 101 kDa were present in the Harderian glands of the intact female and castrated male. Proteolytic products at 51 and 29 kDa in the testosterone-treated female and at 29 kDa in intact male were present.

In their studies with rat liver, Rohde *et al.* (1990) reported a 68 kDa doublet. The thickness and intensity of the 63 kDa band may mask a doublet. In addition to the 63 kDa band, intact female and castrated male Harderian glands showed 54 kDa and 51 kDa bands and a 44 kDa band in intact females; these are likely proteolytic products.

4.2. Use of Densitometry

4.2.1. Introduction

The basis of this thesis was to determine and compare the amounts of ALVS protein in Harderian glands among different experimental groups. Initial analyses were subjective, involving the comparison of the appearance

of bands. ALVS protein concentration was determined by comparing band densities in western immunoblots with ALVS MAb's. It was desirable to obtain a quantitative measure of relative protein amounts; therefore, immunoblot bands were further analyzed using densitometry. This approach is only valid, however, if band density is related to the amount of protein in the band.

The purpose of this experiment was to determine whether a linear relationship exists between protein amount and band density. The approach was to run a range of protein concentrations from the Harderian gland of intact female hamsters, since a stronger signal for ALVS protein was observed with intact female Harderian glands compared to intact male Harderian glands (**Section 4.1**), and then to immunoblot with ALVS MAb. I determined the densities of the observed ALVS bands across this range of different protein amounts and tested to see if there was a linear relationship between the two variables.

4.2.2. Methods

The Harderian gland of a female hamster was dissected and processed for SDS-PAGE and western immunoblotting with ALVS MAb (**Section 3.6.**). Serial dilutions of protein (30 μg, 27 μg, 24 μg, 21 μg, 18 μg, 15 μg, 12 μg, 9 μg, and 6 μg) were loaded on the gel. The immunoblot was then analyzed by densitometry (**Section 3.7.**) and the densities plotted against protein concentration. Correlation coefficients were calculated from these data and tested for significance as outlined in **Appendix E**.

4.2.3. Results

Immunoblotting with ALVS MAb produced a band at 63 kDa in each lane loaded with different amounts of protein (Figure 4.2A). Each 63 kDa band present in each lane was analyzed by densitometry, whereby the optical density was integrated over the entire band area. The densities of the bands were plotted against the total protein amounts (μg) loaded in the lanes (Figure 4.2B). A positive, significant correlation coefficient of 0.96 ($P<0.05$) was obtained between ALVS band density and total protein amount loaded.

4.2.4. Discussion

This experiment was conducted to justify the use and accuracy of densitometry for measuring protein content from immunoblots. Serial dilutions of Harderian gland protein were used; it was assumed that ALVS to total protein ratios would remain constant.

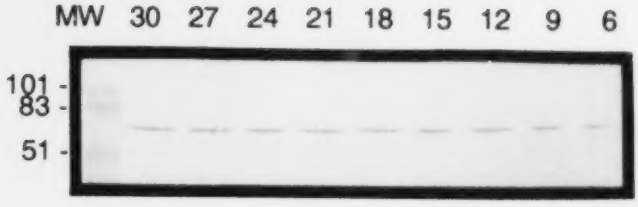
These bands were analyzed by densitometry and the densities plotted versus protein amounts, producing a positive and significant correlation between band density and protein amount. This suggests that band density serves as an adequate measure of relative protein content. Female Harderian gland gives a stronger signal for ALVS protein than does male Harderian gland, in which amounts of ALVS protein are so low that they are near the limits of detection, and the band densities nearly approximate background densities. However, interpolation from Figure 4.2B indicates that ALVS band densities for intact male Harderian glands fall within the protein range of 0-5 μg. While densitometry does not indicate absolute ALVS protein amounts, relative amounts can be established since there is a linear

Figure 4.2.

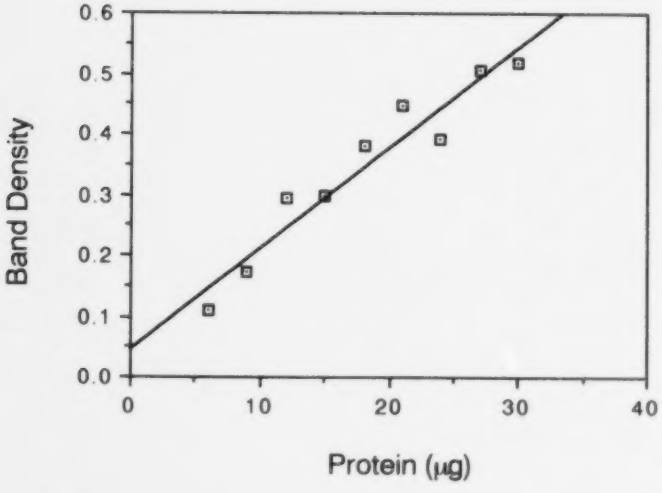
A: Immunoblot of female Harderian gland with different protein amounts (μg) of homogenate loaded in each lane. The numbers at the top of the figure represent the amount of total protein loaded. Immunoblotting with ALVS MAb produced a band at 63 kDa. Molecular weight standards (MW): phosphorylase B (101 kDa), bovine serum albumin (83 kDa), ovalbumin (51 kDa).

B: ALVS band densities-versus-total protein amounts plot showing a positive correlation between band density and protein amount.

A


MW 30 27 24 21 18 15 12 9 6
101 -
83 -
51 -

B


Band Density
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
Protein (µg)

relationship between the amount of ALVS protein and measured band density.

5 REGULATION OF δ-AMINOLEVULINATE SYNTHASE PROTEIN CONCENTRATIONS IN HAMSTER HARDERIAN GLANDS

5.1. Introduction

The Harderian glands of the Syrian hamster show a marked sexual difference in porphyrin production. Female Harderian glands have higher concentrations of porphyrins than male Harderian glands, a difference that could be attributed to greater porphyrin synthesis in females, since ALVS activity and ALVS mRNA have been reported to be higher in females (Thompson *et al.*, 1984; Spike *et al.*, 1990; Menendez-Pelaez *et al.*, 1991; Rodriguez *et al.*, 1993). ALVS activity must be dependent on the amount of enzyme present; however, the concentrations of ALVS protein have not been previously determined.

Previous reports have established that this sexual difference in Harderian porphyrin levels is controlled partly by testosterone, although the complex involvement of other hormones from the pineal, pituitary, and thyroid glands has been suggested (Hoffman, 1971; McMasters and Hoffman, 1984; Buzzell *et al.*, 1989; Hoffman *et al.*, 1990; Buzzell *et al.*, 1992; Buzzell *et al.*, 1994). Despite the proposed involvement of other hormones, testosterone is a major regulator of porphyrin levels. Studies contend that low serum testosterone levels lead to high porphyrin levels; conversely, high serum testosterone levels lead to low porphyrin levels (Marrufo *et al.*, 1989; Buzzell *et al.*, 1991). However, how testosterone maintains low porphyrin levels is unknown.

In hamsters, androgen levels fluctuate in response to the photoperiod.

Despite a lowering of testosterone during short photoperiods, porphyrin levels do not increase (Buzzell *et al.*, 1994). The effects of photoperiod on porphyrin synthesis have not previously been examined.

Therefore, the goals of this study were:

1. to determine whether testosterone affects the concentration of ALVS protein in the Harderian gland; and
2. to determine whether photoperiod modulates ALVS protein concentrations.

The effects of testosterone on ALVS protein concentrations were examined by comparing low and high testosterone groups maintained in a long photoperiod (i.e. intact vs. castrated males; intact vs. testosterone-treated females). To determine whether photoperiod modulates ALVS protein concentrations, females, males, and castrated males were exposed to short or long photoperiods.

5.2. Methods

Female hamsters (treated as described in **Sections 3.1 and 3.2**) were divided into the following groups:

1. intact and maintained in a long photoperiod (Int-LD) (N=6),
2. implanted with a testosterone pellet and maintained in a long photoperiod (Tes-LD) (N=6),
3. intact and maintained in a short photoperiod (Int-SD) (N=6), and
4. pinealectomized and maintained in a short photoperiod (Pnx-SD) (N=5).

Females were sampled when at the same stage (diestrus day 1) of the estrous cycle (**Sections 3.1 and 3.3**) and between nine and nine and a half weeks

after treatment.

Male hamsters (treated as described in **Sections 3.1 and 3.2**) were divided into the following groups:

1. intact and maintained in a long photoperiod (Int-LD) (N=6),
2. castrated and maintained in a long photoperiod (Cas-LD) (N=6),
3. intact and maintained in a short photoperiod (Int-SD) (N=6),
4. castrated and maintained in a short photoperiod (Cas-SD) (N=6), and
5. pinealectomized and maintained in a short photoperiod (Pnx-SD) (N=6).

All male animals were maintained for eight and a half weeks before sampling.

The Harderian glands of female and male animals were dissected, frozen on dry ice, and stored at -70°C until assayed for porphyrin, SDS-PAGE, and western immunoblotting with ALVS MAb (**Sections 3.4 and 3.6**). Immunoblots were analyzed by densitometry (**Section 3.7**).

Data for porphyrin concentrations and ALVS band density are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined using a one-way analysis of variance (ANOVA) to determine whether there were significant between-group differences, followed by a Student-Neuman-Keuls (SNK) test to determine which groups contributed to the differences. Statistics for ANOVA and SNK were calculated manually according to Khazanie (1986) and Kuehl (1994) (**Appendices C and D**).

The existence of a correlation between porphyrin and ALVS protein levels was investigated. For each treatment group, porphyrin levels were

plotted against ALVS band densities using Cricket Graph Version 1.3.2 (Malvern, PA) on a Macintosh Classic (Apple Computer, Inc., Cupertino, CA) computer, which fitted a regression line through the data points and calculated the Pearson product-moment coefficient of linear correlation. Significance was calculated manually according to Khazanie (1986) (**Appendix E**).

5.3. Results

The weight of each animal and reproductive organs were recorded to monitor the effectiveness of the treatments (**Appendix F**). All treatments were effective, with short photoperiod exposure inducing the expected decreases in the weights of the testes and accessory sex glands in males, and of the uterus in females. The decrease in the weight of the accessory sex glands was expected to follow castration.

Immunoblotting with ALVS MAb was conducted on female Harderian glands in all groups (Figures 5.1A-E). ALVS protein was present at 63 kDa in all conditions. The ALVS band was stronger in Int-LD and Pnx-SD than in Tes-LD and Int-SD, and weakest in Tes-LD. Immunoblots were analyzed by densitometry and the average porphyrin concentrations for each group (Figure 5.2A) were plotted for comparison against the average ALVS band density for each group (Figure 5.2B).

In comparison to the control (Int-LD), testosterone treatment and short photoperiod significantly decreased porphyrin levels and ALVS band densities. Pinealectomy appeared to reduce the decreases (of porphyrin levels and ALVS band densities) induced by short day exposure. In addition, pinealectomy significantly increased ALVS band density

Figure 5.1.

Immunoblot analysis of ALVS protein in Harderian glands of intact (Int), testosterone-treated (Tes), and pinealectomized (Pnx) female hamsters exposed to a long (LD) or short (SD) photoperiod. ALVS protein was present at 63 kDa in all blots. Molecular weight standards (MW): phosphorylase B (112 kDa), bovine serum albumin (84 kDa), ovalbumin (53 kDa).

A: ALVS protein levels compared among all groups: Tes-LD, Int-LD, Int-SD, and Pnx-SD. The ALVS band density was stronger in Int-LD and Pnx-SD than in Tes-LD and Int-SD, and weakest in Tes-LD.

B-E: ALVS protein levels compared among individuals of the same group.

B: Tes-LD

C: Int-LD

D: Int-SD

E: Pnx-SD

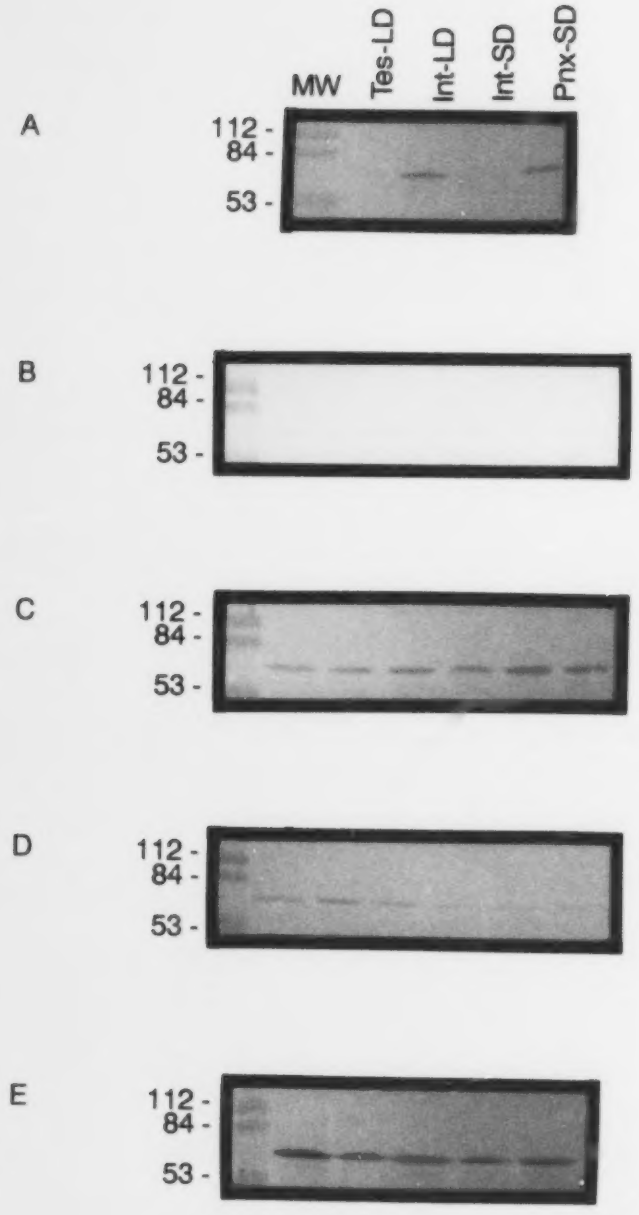

MW
Tes-LD
Int-LD
Int-SD
Pnx-SD
A
112 -
84 -
53 -
B
112 -
84 -
53 -
C
112 -
84 -
53 -
D
112 -
84 -
53 -
E
112 -
84 -
53 -

Figure 5.2.

A: Porphyrin concentrations (mean ± SEM) of Harderian glands of intact (Int), testosterone-treated (Tes), and pinealectomized (Pnx) female hamsters exposed to a long (LD) or short (SD) photoperiod. In comparison to the control (Int-LD), testosterone treatment (Tes-LD) and short-day exposure (Int-SD) decreased porphyrin levels. All groups were significantly different from each other with $P<0.05$ (ANOVA, SNK).

B: ALVS band densities (mean ± SEM) of Harderian glands of females in the same treatments as described for Figure 5.2A. Testosterone treatment and short days decreased ALVS band density compared to the control and pinealectomy. Pinealectomy increased ALVS band density compared to the control. In the control and pinealectomized groups, ALVS band densities did not parallel the levels of porphyrins. Significance: *, $P<0.05$ vs. all groups (ANOVA, SNK).

These figures show that pinealectomy (Pnx-SD) appeared to reduce the decreases (in porphyrin concentration and ALVS band density) induced by exposure to a short photoperiod.

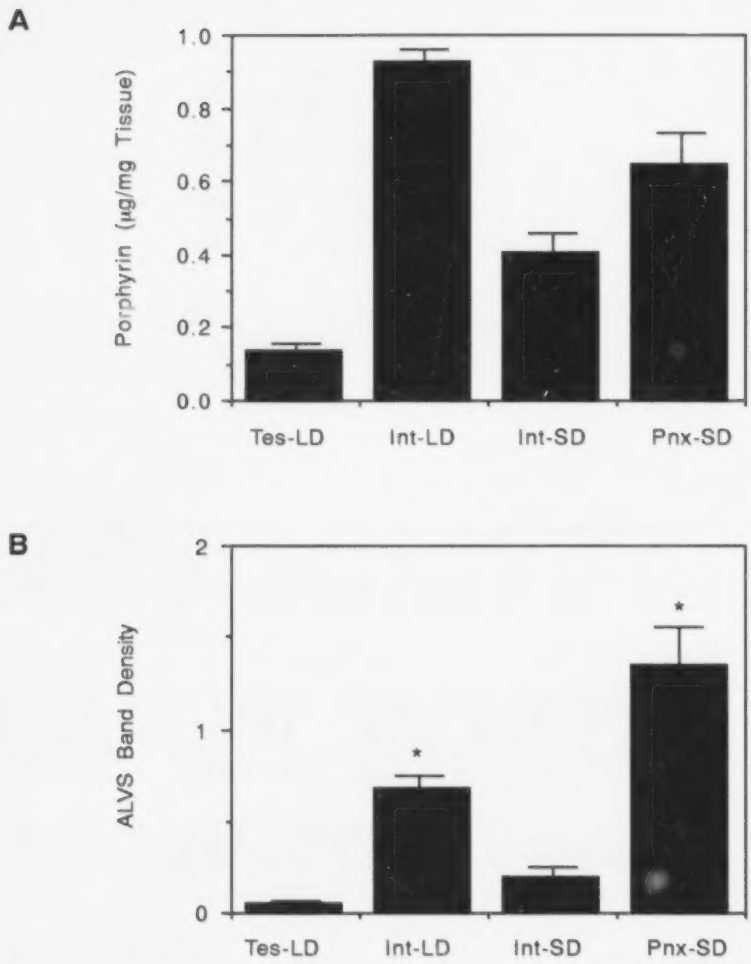

A
Porphyrin (µg/mg Tissue)
1.0
0.8
0.6
0.4
0.2
0.0
Tes-LD
Int-LD
Int-SD
Pnx-SD
B
ALVS Band Density
2
1
0
*
*
Tes-LD
Int-LD
Int-SD
Pnx-SD

Figure 5.3.

Immunoblot analysis of ALVS protein in Harderian glands of intact (Int), castrated (Cas) and pinealectomized (Pnx) male hamsters exposed to a long (LD) or short (SD) photoperiod. ALVS protein was present at 63 kDa. Molecular weight standards (MW): phosphorylase B (112 kDa), bovine serum albumin (84 kDa), ovalbumin (53 kDa).

A: ALVS protein levels compared among all groups: Cas-LD, Int-LD, Int-SD, Cas-SD, and Pnx-SD. The ALVS band density was strongest in Cas-LD.

B-F: ALVS protein levels compared among individuals of the same group.

B: Cas-LD

C: Int-LD

D: Int-SD

E: Cas-SD

F: Pnx-SD

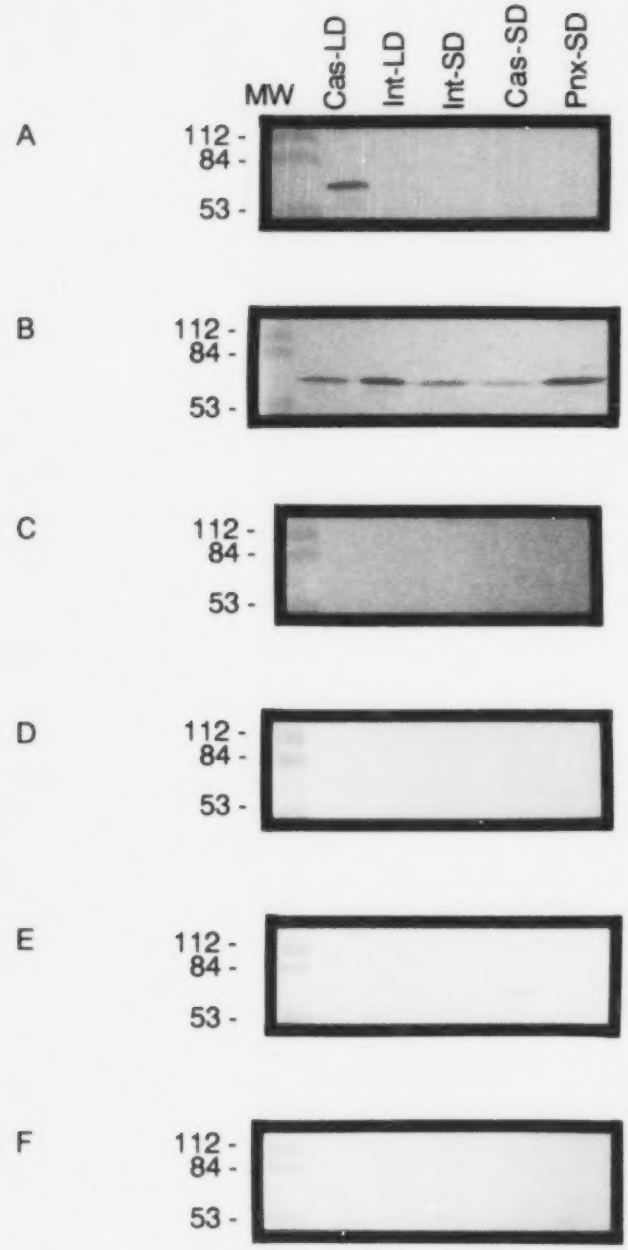

MW
Cas-LD
Int-LD
Int-SD
Cas-SD
Pnx-SD
A
B
C
D
E
F
112 -
84 -
53 -

Figure 5.4.

A: Porphyrin concentrations (mean ± SEM) of Harderian glands of intact (Int), castrated (Cas) and pinealectomized (Pnx) male hamsters exposed to a long (LD) or short (SD) photoperiod. Castration (Cas-LD) increased porphyrin levels compared to the control (Int-LD) and other groups, in which porphyrin concentrations were low. Short photoperiod exposure (Cas-SD) prevented the rise in porphyrin levels. Pinealectomy and maintenance in short days (Pnx-SD) had no effect on porphyrin concentrations. Significance: *, $P<0.05$ vs. other groups (ANOVA, SNK).

B: ALVS band densities (mean ± SEM) of Harderian glands of males in all treatments as described in Figure 5.4A. Treatments had the same effects on ALVS protein concentrations as with porphyrin levels (described above). Significance: *, $P<0.05$ vs. other groups (ANOVA, SNK).

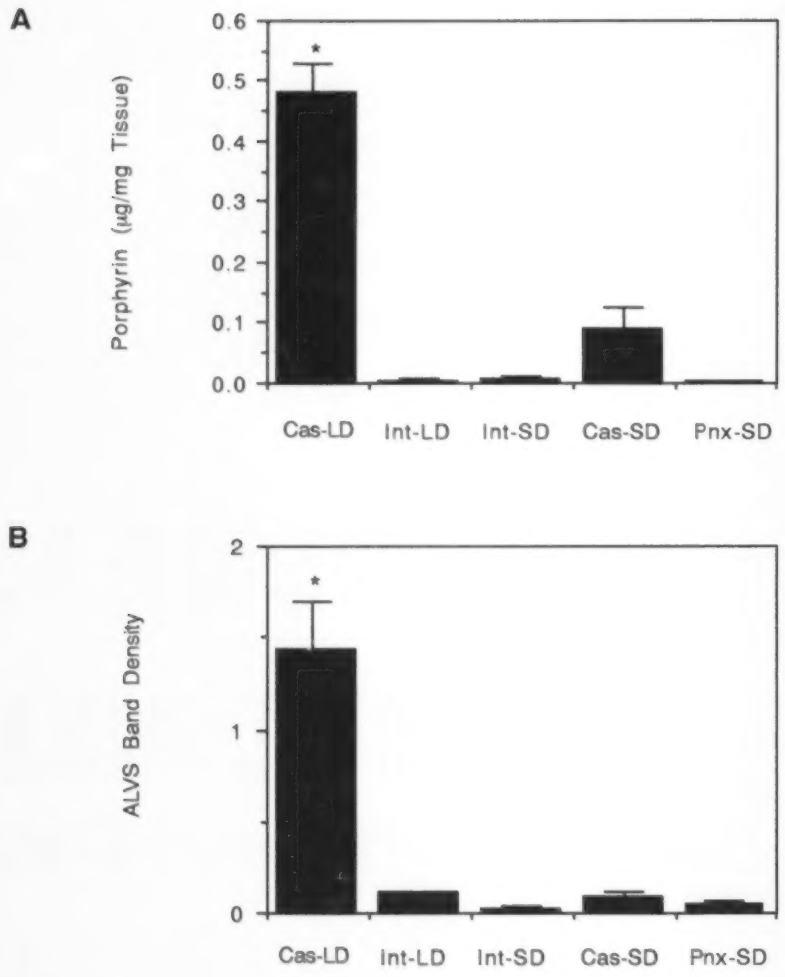

A
Porphyrin (µg/mg Tissue)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Cas-LD
Int-LD
Int-SD
Cas-SD
Pnx-SD
B
ALVS Band Density
2
1
0
*
Cas-LD
Int-LD
Int-SD
Cas-SD
Pnx-SD

Table 5.1.

Relationship between ALVS Protein Concentrations and Porphyrin Levels

Group	n	Coefficient of Linear Correlation
Female		
Tes-LD	**6**	- 0.40
Int-LD	**6**	+ 0.10
Int-SD	**6**	+ 0.18
Pnx-SD	**5**	- 0.008
Male		
Cas-LD	**6**	- 0.05
Int-LD	**6**	- 0.74
Int-SD	**6**	+ 0.76 *
Cas-SD	**6**	+ 0.77 *
Pnx-SD	**6**	+ 0.89 *

Significance: *, $P<0.05$

compared to the control. In the control and pinealectomized groups, changes in ALVS band densities did not correspond to the changes in the levels of porphyrins.

Immunoblotting with ALVS MAb was conducted on male Harderian glands in all groups (Figures 5.3A-F). The ALVS band appeared strongest in Cas-LD. Immunoblots were analyzed by densitometry and the average porphyrin levels for each group (Figure 5.4A) were plotted to compare against the average ALVS band density for each group (Figure 5.4B).

Castration (Cas-LD) significantly increased porphyrin levels and ALVS band densities compared to the control (Int-LD) and all other groups. Short photoperiod exposure (Cas-SD) prevented these rises in porphyrin levels and ALVS band density. In uncastrated hamsters, pinealectomy and short day exposure had no effects on porphyrin concentrations and ALVS band density, which were similar to those of the control.

Porphyrin levels (μg/mg tissue) and corresponding ALVS band densities for individuals of each treatment group were plotted and coefficients of linear correlation were calculated for each treatment group (Table 5.1). No linear correlation was found between porphyrin levels and ALVS protein concentrations in all female groups. Among the male groups, significant linear correlations were obtained for the short photoperiod treatments but not in the long photoperiod treatments.

5.4. Discussion

The Harderian glands of the Syrian hamster show a marked sexual difference in porphyrin production, with female Harderian glands having higher concentrations of porphyrins compared to males (Moore *et al.* 1977;

Thompson *et al.*, 1984). ALVS, the rate-limiting enzyme of the heme synthesis pathway, is subject to negative feedback regulation by heme (Padmanaban *et al.*, 1989). ALVS activity and ALVS mRNA have been reported to be higher in females than in males (Thompson *et al.*, 1984; Spike *et al.*, 1990; Menendez-Pelaez *et al.*, 1991; Rodriguez *et al.*, 1993). These studies imply that the sexual difference in porphyrin levels may be associated with greater porphyrin synthesis in females compared to males, assuming that ferrochelatase (the enzyme that metabolizes porphyrin into heme) exhibits relatively low enzymatic activity in both sexes (Thompson *et al.*, 1984).

It has not been previously determined whether high levels of porphyrin synthesis correspond to high amounts of ALVS protein. In addition to higher ALVS mRNA and activity, the concentrations of ALVS protein are likely greater in females than in males. My results have shown that the amount of enzyme was indeed higher in intact females than in intact males.

My work showed that porphyrin levels did not correlate with ALVS protein amounts. No linear correlation between ALVS protein concentrations and porphyrin levels was observed in females. In males, a significant linear correlation was observed in the short photoperiod, but not in the long photoperiod groups. A possible explanation for the significant linear correlations is that the values for porphyrin and ALVS protein levels were close to zero in male Harderian glands and were near the lower limits of detection for the techniques used in this study. The lack of linear correlation suggests that the levels of porphyrins are possibly dependent upon the amount of enzymatically active ALVS rather than total ALVS

protein concentrations.

Data from pinealectomized females also indicated that there is no relationship between ALVS protein concentrations and porphyrin levels. Pinealectomized females exposed to a short photoperiod had double the amount of ALVS protein but lower levels of porphyrins compared to the control females (intact females maintained in a long photoperiod). Several interpretations can be drawn from these results.

The lack of a correlation between porphyrin levels and ALVS protein amounts could be due to the techniques used to assay porphyrin and ALVS protein levels. Female Harderian glands have high porphyrin levels and contain large, intraluminal porphyrin deposits, which consist of stored, inert porphyrin. The problem with interpreting porphyrin results is the difficulty in distinguishing what proportion of measured porphyrin represents inert, stored porphyrin (e.g. the deposits characteristic of female glands); newly formed porphyrin; or porphyrin which is being mobilized for some specific function. By contrast, western blotting measures only total amounts of protein and these proteins may not necessarily be enzymatically active. The absence of a correlation between porphyrin levels and ALVS protein concentrations may reflect the limitations of these techniques.

In pinealectomized females maintained in a short photoperiod, the high ALVS protein concentration is a reflection of total ALVS protein, but not necessarily active protein; therefore, this could result in the observed lower porphyrin levels. Enzyme activity is dependent upon the availability of the substrates, glycine and succinate, and the cofactor, pyridoxal phosphate. These substrates and cofactors themselves are generated from sources which may be limited. If these sources, substrates, or cofactors are

limited, then it is likely that the ALVS protein concentrations could be much higher than porphyrin levels.

Alternatively, it is possible that ALVS is active, but not as much porphyrin is produced due to inhibition of one of the other enzymes or intermediates downstream in the heme synthesis pathway. Inhibitors could prevent the transformation of substrates into porphyrin, or substrates and/or porphyrin could be excreted from the cell.

Furthermore, one could speculate that all the ALVS protein is active, but the porphyrin that is produced is either secreted to the exterior of the gland or degraded into heme or hemoproteins by ferrochelatase.

Another possibility is illustrated by comparing intact females and pinealectomized females both exposed to a short photoperiod. A short photoperiod decreased porphyrin levels and ALVS protein concentrations. Pinealectomy appeared to reduce the decrease in porphyrin concentration induced by short day exposure. With pinealectomy, perhaps some unknown compensation mechanism tries to reduce this decrease by producing more ALVS protein, which may not be all active; consequently, lower porphyrin levels than expected are observed.

Previous studies have established that the sexual difference in porphyrin concentrations is regulated partly by testosterone, since castration of males increases porphyrin levels, while testosterone treatment of females lowers them (Marrufo *et al.*, 1989; Buzzell *et al.*, 1991). With regard to ALVS, castration of males increases ALVS mRNA and ALVS activity levels, and testosterone treatment of females decreases them (Spike *et al.*, 1990; Menendez-Pelaez *et al.*, 1991).

My work also showed that castration of males (those exposed to a

long photoperiod) increased ALVS protein concentrations, while testosterone treatment of females decreased them. These findings suggest that testosterone reduces the amount of ALVS protein. As discussed previously, porphyrin levels do not correlate with ALVS protein concentrations. Consequently, testosterone likely decreases ALVS protein concentrations, resulting in less ALVS activity and hence, reduced porphyrin levels.

Interestingly, castrated males (those maintained in a long photoperiod) had twice as much ALVS protein as intact females kept in a long photoperiod. Females have high activity of aromatase, the ovarian enzyme which converts testosterone into estradiol (Voet and Voet, 1995). A homeostatic balance is maintained between estradiol and testosterone, such that high levels of estradiol are formed, but still low levels of testosterone remain. Males have low activity of aromatase; therefore, testosterone is the major hormone and is not likely to be converted into estradiol. The male adrenal glands produce low levels of testosterone, with larger quantities being produced by the testes; however, castration has a drastic effect on testosterone levels, reducing them nearly to nil. An explanation as to why castrated males had twice as much ALVS protein as intact females kept in a long photoperiod, could be that females still have low amounts of circulating testosterone, but the consequences of removing testosterone from males has a more dramatic effect, elevating ALVS protein concentrations. Higher ALVS protein concentrations could allow for higher ALVS activity; thus, porphyrin levels increase in turn.

It has been proposed that other hormones, besides testosterone, also influence ALVS protein concentrations. This was verified by the results of

the short photoperiod groups.

Long photoperiods (more than 12.5 hours per day) are required to maintain gonadal size and function in hamsters (Reiter, 1973). In contrast, short photoperiods decrease levels of sex steroids, gonadotrophin, prolactin, and thyroid hormones, leading to reproductive quiescence. Removal of the pineal gland prevents reproductive quiescence. The effects of short days are controlled by the pineal gland, since hamsters are sexually functional in all photoperiods when they are pinealectomized (Reiter, 1980).

Upon exposure to a short photoperiod, intact males have low testosterone levels compared to those of males maintained in long days (Buzzell *et al.*, 1995). With low testosterone, one would expect an increase in porphyrin concentrations. Nevertheless, it has been reported that short photoperiod decreases testosterone levels with no increase in porphyrin concentrations (Buzzell *et al.*, 1994). Likewise, my work showed that a short photoperiod had no effect on ALVS protein concentrations in males.

Comparison of the castrated groups further supports the involvement of a "short photoperiod factor." Castrated males maintained in long days show increased porphyrin levels; yet, castrated males kept in short days had normal porphyrin levels (Buzzell *et al.*, 1994). In my work, ALVS protein concentrations of castrated males kept in long days differed from those of castrated males exposed to a short photoperiod. Castrated males kept in long days showed high ALVS protein levels, presumably due to low testosterone. In contrast, castration and exposure to short days had no effect. Short photoperiod therefore prevented the rise in ALVS protein amounts that might be expected to follow castration or low testosterone levels.

These findings indicate that some aspect of short photoperiod

maintains low ALVS protein amounts in males (hence low ALVS activity and low porphyrin levels), even though serum testosterone levels are normally decreased by short photoperiod or eliminated by castration.

Previous studies suggest that the factor regulating the effects of short photoperiod is produced by the pineal gland (Reiter, 1980). The pineal gland influences not only the reproductive system but also the Harderian gland. Castrated males maintained in a long photoperiod showed Harderian gland morphology similar to that of females. Conversely, blind castrated males maintained the male Harderian gland morphology; however, with pinealectomy, blind castrated males had female Harderian gland characteristics (Hoffman, 1971; Clabough and Norvell, 1973). These observations imply that the pineal gland enables the Harderian gland to respond appropriately to changing photoperiods.

It has been reported that Harderian porphyrin levels in pinealectomized castrated males maintained in short days were high, just as those observed in castrated males kept in a long photoperiod (Buzzell *et al.*, 1994); in contrast, non-pinealectomized castrated males kept in a short photoperiod had low porphyrin levels, similar to intact males in a long photoperiod (Buzzell *et al.*, 1994).

There is no evidence in my work to confirm that the pineal gland controls ALVS concentrations in male Harderian glands, since concentrations of ALVS protein in castrated, pinealectomized males kept in short days were not investigated. The involvement of the pineal gland in males would be further substantiated if high ALVS protein levels were to be found in this group.

On the contrary, this study provided evidence of pineal gland

involvement in female Harderian glands. In females, short photoperiod decreased ALVS protein concentrations (limiting ALVS activity), and resulted in low porphyrin levels. Pinealectomy appeared to prevent most of the decrease in porphyrin concentrations induced by short photoperiod exposure and elevated ALVS protein levels (for reasons which were discussed earlier).

The effects of the pineal gland are not usually mediated directly by the pineal hormone, melatonin, but by melatonin's influence on the production and secretion of other hormones. Previous studies suggest that the effects of short days on Harderian gland porphyrin concentrations are controlled by some hormone which itself is regulated by melatonin. This hormone may be prolactin (Buzzell *et al.*, 1989, 1992, 1995; Marr *et al.*, 1995).

Secretion of prolactin from the pituitary gland is inhibited by dopamine, a hormone of the hypothalamus. An ergot alkaloid, bromocriptine is a dopamine agonist which suppresses prolactin secretion, reducing circulating prolactin levels. Bromocriptine had no effect on porphyrin levels in intact males. However, in castrated males, bromocriptine prevented the rise in porphyrins that normally follows castration. Administration of prolactin reversed the effects of bromocriptine and increased porphyrin levels in castrated males (Marr *et al.*, 1995; Buzzell *et al.*, 1989). Prolactin also influenced ALVS activity. Bromocriptine administrated to castrated males prevented the rise in ALVS activity that would be expected to follow castration. This effect was reversed by treatment with prolactin, showing this hormone to be necessary in a low testosterone milieu to stimulate porphyrin synthesis (Marr *et al.*, 1995).

My work confirmed that testosterone affects ALVS protein amounts, although the possible influence of prolactin on ALVS protein concentrations can only be inferred by analogy with the situation of Harderian gland porphyrin concentrations. In a low testosterone environment, prolactin may stimulate ALVS protein synthesis; thus, in castrated males, ALVS protein concentrations are high. Consequently, ALVS activity may be raised due to the presence of more enzyme, and porphyrin levels will elevate.

Conversely, in a low testosterone environment with reduced serum prolactin levels (e.g. short days), porphyrin and ALVS protein concentrations are low. Intact and castrated males exposed to a short photoperiod had low porphyrin and ALVS protein concentrations. In short days, females have low prolactin and testosterone levels. It was observed that females kept in a short photoperiod had lower porphyrin and ALVS protein concentrations compared to intact females. In a short photoperiod, prolactin is at a level which is too low to induce porphyrin synthesis. As a result, ALVS protein concentrations remain low. The absence of high enzyme concentrations leads to lower ALVS activity, hence low porphyrin levels.

In summary, my work confirmed that testosterone influences ALVS protein concentrations. A factor of short photoperiod is involved in regulating ALVS protein concentrations, although my results did not confirm whether this factor involves the pineal gland, as proposed by other reports. Previous studies have implied that the sexual difference in porphyrin levels in hamster Harderian glands is mediated by greater porphyrin synthesis, as suggested by higher ALVS activity and ALVS

mRNA levels in females compared to males. ALVS activity must be dependent upon the amount of enzyme present. However, ALVS protein concentrations had not been previously investigated and were addressed in my work. It was hypothesized that higher porphyrin levels in females would be associated with higher ALVS protein concentrations; however, data from my work rejects this hypothesis. Porphyrin levels and ALVS protein concentrations were not correlated. I conclude from this finding that levels of porphyrin are actually correlated to the amount of enzymatically active ALVS rather than to the total amount of ALVS protein present. Active, rather than total enzyme, may be the significant factor in regulating porphyrin levels.

6 δ-AMINOLEVULINATE SYNTHASE IN OTHER RODENT SPECIES

6.1. Introduction

Many rodent Harderian glands contain high concentrations of porphyrins, while others may not. For the purpose of comparison with Syrian hamsters, porphyrin and ALVS protein concentrations of other rodent species were investigated. These include the blind mole rat (*Spalax ehrenbergi*) and two species of spiny mouse of the genus *Acomys* (*A. russatus* and *A. cahirinus*).

The blind mole rat is endemic to northern and central Israel (Rado *et al.*, 1989). It is a solitary, highly aggressive rodent which has adapted well to its subterranean environment (Rado *et al.*, 1989). Its cylindrical, elongate body, short fur, and absence of bodily protrusions (such as a tail and ears), enable its free movement through tunnels. The eyes (each less than 0.5 mm in diameter) are atrophied and covered by a thick layer of skin, and consequently are not detectable externally. Each eye rests on a large Harderian gland.

Regardless of a degeneration of the visual system, the atrophied eyes of the blind mole rat have maintained connections with the suprachiasmatic nuclei, enabling the animal to respond to photoperiod (Rado *et al.*, 1991).

It has been suggested that the Harderian gland in the blind mole rat is involved in light detection, indole metabolism, and pheromone production (Balemans *et al.*, 1980; Pevet *et al.*, 1984; Shanas *et al.*, 1991; Shanas *et al.*, 1995). In Syrian hamsters, Harderian porphyrins and ALVS protein concentrations are influenced by photoperiod. Blind mole rats prefer dark

environments (Rado *et al.*, 1991); therefore, it was of interest to examine Harderian porphyrin and ALVS protein concentrations in a rodent whose light exposure is restricted.

Spiny mice are distributed throughout Asia Minor and West and East Africa (Bates, 1994). The common spiny mouse, *A. cahirinus*, is found in desert and rocky habitats, while the golden spiny mouse, *A. russatus*, is restricted to extremely arid, desert regions. Both species are nocturnal. However, the common spiny mouse remains nocturnal, while the golden spiny mouse becomes diurnal in regions where they coexist (Kronfeld *et al.*, 1994). The Harderian glands of coexisting spiny mice have been found to differ histologically, indicating that diurnal and nocturnal spiny mice have adapted differently to their environments (Haim and Shanas, 1994).

Porphyrin concentrations in Syrian hamster Harderian glands can be regulated by the photoperiod (Buzzell *et al.*, 1994). As shown previously, ALVS protein concentrations are influenced by photoperiod. Porphyrin and ALVS protein concentrations have not been previously investigated in the blind mole rat and spiny mouse. Unlike the blind mole rats which restrict themselves to subterranean environments, spiny mice are exposed to light since they are non-burrowing. These animals are thus of interest since they are exposed to different patterns of environmental light compared to those of the Syrian hamster.

6.2. Methods

The Harderian glands of the blind mole rats were prepared by U. Shanas (Department of Zoology, University of Tel Aviv), while those of the spiny mice were prepared by Dr. A. Haim (Department of Biology,

University of Haifa-Oranim). The porphyrin assays were performed by Dr. G.R. Buzzell (Department of Anatomy and Cell Biology, University of Alberta).

Since mole rats do not breed in captivity, all adult animals were captured in the Tel Aviv area. They were maintained in the laboratory at 24 ± 2 °C and exposed to 14 hours of light and 10 hours of darkness daily.

Harderian glands were dissected from four anesthetized mole rats. Glands from two females and two males were removed during summer (August-September) and autumn (November). Glands were frozen and stored at -70 °C until they were transported to Canada where they were assayed.

Two species of spiny mouse, the common spiny mouse, *A. cahirinus*, and the golden spiny mouse, *A. russatus*, were from a colony kept in Oranim, Israel. Two males and two females of each species were used. Animals kept in the laboratory were exposed to either long day photoperiods (16 hours of light and 8 hours of darkness daily) or short day photoperiods (8 hours of light and 16 hours of darkness daily) at 27 ± 1 °C for 3 weeks before sampling. Harderian glands were dissected, frozen, and stored at -70 °C until they were transported to Canada where they were assayed.

Harderian glands were processed for porphyrin determination (**Section 3.4**), SDS-PAGE and western immunoblotting with ALVS MAb (**Section 3.6**). Immunoblots were analyzed using densitometry (**Section 3.7**).

6.3. Results

No porphyrin was detectable in the glands of the blind mole rats and spiny mice. Immunoblotting with ALVS MAb was conducted on all species (Figures 6.1 and 6.2) and the 63 kDa band (mature ALVS protein) was analyzed by densitometry (Table 6.1).

In both sexes of the blind mole rats, a faint band was present at 74 kDa and stronger bands at 63 and 46 kDa (Figure 6.1). The amount of the 74 kDa protein was similar in all groups. The amount of the 63 kDa protein was higher in males than in females, and higher in the short photoperiod (autumn) than in the long photoperiod (summer) for both sexes (Table 6.1). In both sexes, the amount of the 46 kDa protein was higher in the summer than in the autumn.

In the common spiny mouse (*A. russatus*), the 63 kDa band was present in all groups (Figure 6.2). The amount of the 63 kDa protein did not differ between the sexes (Table 6.1). A 53 kDa band was more obvious in males and very faint in the female kept in long days. A 49 kDa band was present in all groups, being more intense in the short photoperiod groups. Bands at 31 and 29 kDa are likely proteolytic products.

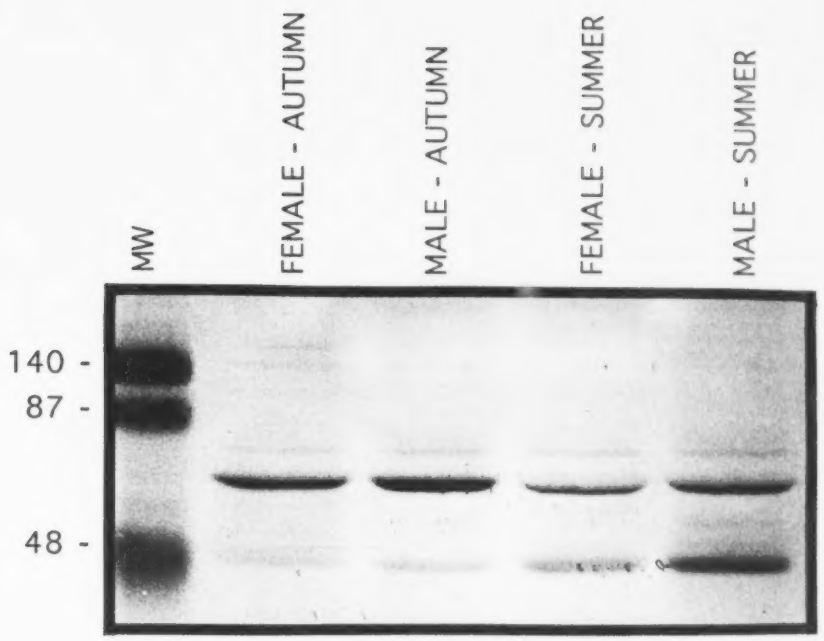
In the golden spiny mouse (*A. cahirinus*), the 63 kDa band was present in all groups (Figure 6.2). The amount of the 63 kDa band was higher in females than in males, and higher in a long photoperiod compared to a short photoperiod (Table 6.1). Bands at 53 and 49 kDa were present in all groups and were more intense in females and in long photoperiods.

6.4. Discussion

For comparison with Syrian hamsters, porphyrin and ALVS protein

Figure 6.1.

Immunoblot analysis of ALVS protein in the blind mole rat (*Spalax ehrenbergi*). Females and males were sampled in either autumn or summer. In both sexes, a faint band at 74 kDa and stronger bands at 63 and 46 kDa were present. The amount of the 74 kDa protein appeared similar in all groups. In both sexes, the amount of the 63 kDa protein was higher in a short photoperiod (autumn), whereas the amount of the 46 kDa protein was higher in a long photoperiod (summer).



Spalax ehrenbergi

Figure 6.2.

Immunoblot analysis of ALVS protein in two species of spiny mouse, *A. russatus* and *A. cahirinus*. Females and males were exposed to long and short photoperiods. In *A. russatus*, bands at 63 kDa were present in all groups. A 53 kDa band was more obvious in males and very faint in the female kept in a long photoperiod. A 49 kDa band was present in all groups, being more intense in the short photoperiod groups. Proteolytic products at 31 and 29 kDa were observed. In *A. cahirinus*, a 63 kDa band was present in all groups, with the band being stronger in a long photoperiod than in a short photoperiod. It was also darker in females than in males. Bands at 53 and 49 kDa were present in all groups and were more intense in long photoperiods and in females.

Acomys

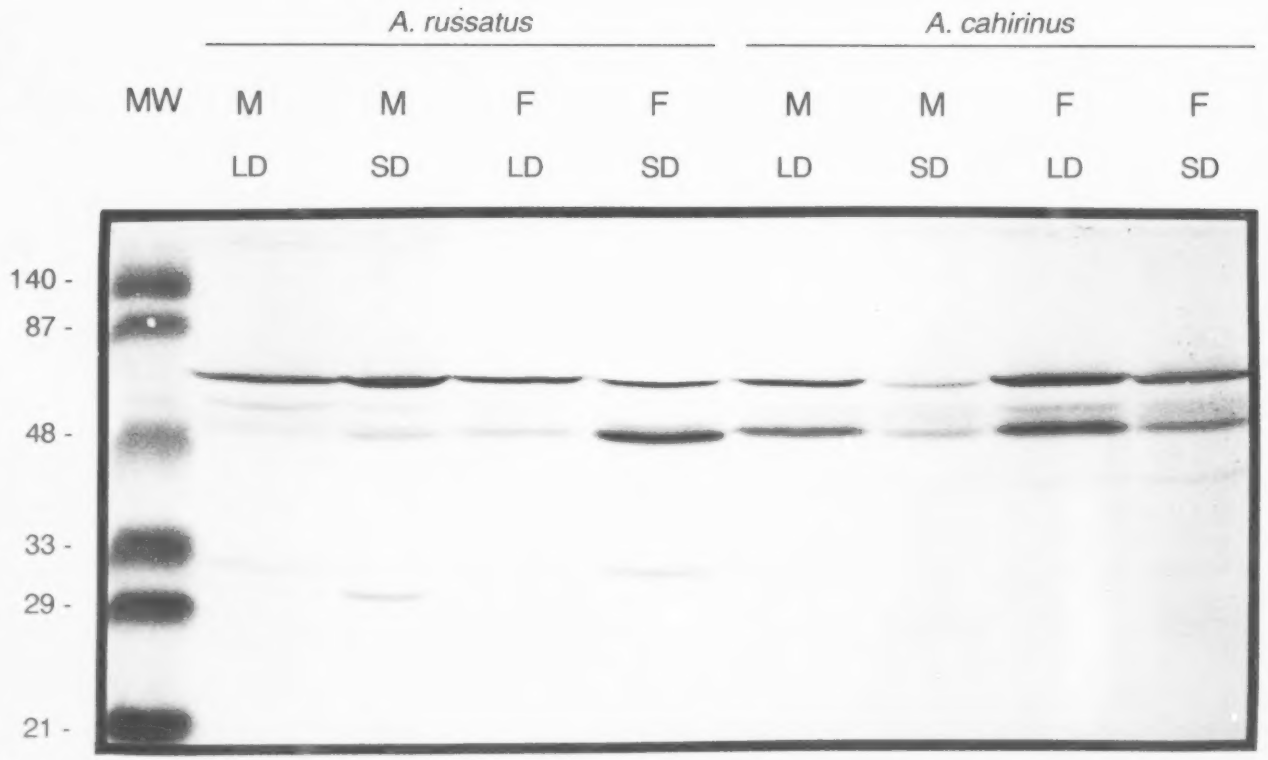

A. russatus
A. cahirinus
MW
M
M
F
F
M
M
F
F
LD
SD
LD
SD
LD
SD
LD
SD
140 -
87 -
48 -
33 -
29 -
21 -

Table 6.1.
ALVS Band Density (63 kDa) in Blind Mole Rats and Spiny Mice

Group	n	ALVS Band Density
Spalax ehrenbergi		
Male - Autumn	1	0.77
Male - Summer	1	0.52
Female - Autumn	1	0.52
Female - Summer	1	0.39
A. russatus		
Male - LD	1	0.73
Male - SD	1	1.32
Female - LD	1	0.84
Female - SD	1	0.75
A. cahirinus		
Male - LD	1	0.84
Male - SD	1	0.34
Female - LD	1	1.16
Female - SD	1	0.63

concentrations of the blind mole rats and spiny mice were determined. No porphyrin was detected in all three species. However, ALVS protein was present in relatively high concentrations. This suggests that the glands produce porphyrin which is metabolized into heme or hemoproteins which have not yet been identified. Conversely, the porphyrin might be secreted externally from the gland. Release of porphyrin outside the gland could be possible in the blind mole rat, which extrudes Harderian gland secretions from the conjunctival sac to the nasal cavity during autogrooming (Shanas *et al*., 1991; Shanas *et al*., 1995; Shanas *et al*., 1996). Another possibility is that ALVS is not active in these animals, or that in these species the glands are vestigial.

In Syrian hamsters, the ALVS protein concentration is higher in females than in males. In comparison, in the blind mole rats, the concentration of ALVS protein was higher in males than in females. In addition, the amount of ALVS protein in both sexes was higher in a short photoperiod (autumn) than in a long photoperiod (summer). In a long photoperiod, the amount of a 46 kDa protein was greater than in a short photoperiod. This result suggests that in a long photoperiod, ALVS protein is metabolized, resulting in a greater concentration of a breakdown product, namely the 46 kDa protein.

In blind mole rats, ALVS protein concentration is higher with exposure to short, rather than long, photoperiods. This may be of some adaptive significance. Israel's climate exhibits two distinct "seasons:" a rainy winter (November to April) and a dry summer (May to October) (Nevo, 1991). Abundant rain in winter softens the soil (facilitating burrowing) and provides vegetation, while dryness in summer has opposite

effects, posing physical and physiological strains on the animal. One may speculate that in long photoperiods, the environmental stresses are such that synthesis of ALVS protein is curtailed, any ALVS protein remaining degrades, and energy is diverted to other physiological systems.

In *A. russatus*, there were no discernible differences (based on gender and photoperiod) on ALVS protein concentrations. A 49 kDa protein appeared to be more intense in short photoperiods in both sexes. Presumably, the 49 kDa protein is a proteolytic product. There was no correspondence in ALVS protein concentrations (based on gender and photoperiod) between *A. russatus* and the Syrian hamster.

On the contrary, *A. cahirinus* exhibited a pattern very similar to Syrian hamsters. *A. cahirinus* females had higher ALVS protein concentrations than males, a dimorphism that is evident also in Syrian hamsters. Exposure to a short photoperiod decreased ALVS protein concentrations in *A. cahirinus* females, an effect which was observed in female Syrian hamsters. ALVS protein concentrations also decreased in *A. cahirinus* males exposed to short-days, although this effect was not observed with male Syrian hamsters. An explanation for the similarities in ALVS protein concentrations between the two species could be that both are nocturnal; hence, they respond similarly to fluctuating photoperiods.

To summarize, ALVS protein concentrations were similar in certain conditions between Syrian hamsters and *A. cahirinus*. No corresponding similarities were observed with any of the other species. Presumably, these differences among dissimilar species may reflect their individual adaptations to the distinct environments which they inhabit.

7 GENERAL CONCLUSIONS

The sexual dimorphism in porphyrin levels of female and male Syrian hamsters is thought to be due to greater ALVS mRNA and ALVS activity levels in females. However, ALVS protein concentrations had not been previously investigated. I examined whether the sexual dimorphism in porphyrin levels can be attributed to corresponding differences in ALVS protein concentrations. Female Harderian glands had higher ALVS protein concentrations than males, suggesting that the sexual difference in porphyrin levels is associated with greater ALVS protein concentrations in females.

Porphyrin concentrations are partly controlled by androgens, since castration of males leads to an increase in porphyrin levels, whereas testosterone treatment of females leads to their decrease. Castrated males had higher ALVS protein amounts than intact males, whereas testosterone-treated females showed lower ALVS protein concentrations than intact females. These findings suggest that ALVS is regulated in part by testosterone.

In addition, the effect of photoperiod (which also influences porphyrin concentrations) on ALVS protein concentrations was examined. The results observed with short photoperiod implicated the involvement of another hormone, possibly prolactin, in mediating ALVS protein amounts.

From these findings, the amount of ALVS protein was suspected to be an indicator of the levels of porphyrin; hence, it was further investigated whether ALVS protein concentrations correspond with porphyrin levels. Porphyrin levels did not correlate well with ALVS protein amounts, leading

to the conclusion that active, rather than total ALVS enzyme, is significant in mediating porphyrin levels.

Other rodent species were examined to determine whether porphyrin and ALVS protein concentrations (based on gender and photoperiod) in these species are comparable to those of the Syrian hamster. However, no porphyrin was detectable in the Harderian glands of the blind mole rat and both species of spiny mice, but ALVS protein was present, suggesting that any porphyrin that is formed is converted into heme or hemoproteins. Ferrochelatase, the enzyme that converts protoporphyrin into heme, may thus be present and active in these species. Other possibilities may be that porphyrin is secreted to the exterior of the gland; that ALVS protein is not active in these animals; or that the gland is vestigial in these species.

The blind mole rat (a subterranean animal) and *A. russatus* (a diurnal species) showed no similarities in ALVS protein concentrations; in comparison, *A. cahirinus* and Syrian hamsters exhibited similarities in ALVS protein amounts, presumably reflecting their nocturnal lifestyle. These findings indicate that the different species have adapted accordingly to the rigors of their specific environments.

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APPENDICES

Appendix A: Protein Assay

Protein content was measured according to the method of Lowry *et al.* (1951).

Stock solutions:

1 N NaOH

2% Na_2CO_3

1% $CuSO_4 5H_2O$

2% Sodium tartrate

1 N Folin's reagent

0.15% BSA

Copper reagent prepared fresh (for 50 mL): 2% Na_2CO_3 (48 mL)
2% Sodium tartrate (1 mL)
1% $CuSO_4 5H_2O$ (1 mL)

Standards to prepare:

Number	Stock (μL)	Protein (mg)
B	0	blank
1	10	0.015
2	20	0.030
3	40	0.060
4	60	0.090
5	80	0.120
6	100	0.150
7	120	0.180
8	150	0.225

Procedure:

1. Prepare blanks and standards in duplicate.
2. Prepare samples in duplicate. For Harderian glands, use 10-20 μL of homogenate. Bring up to 150 μL with distilled water.
3. Add 200 μL of 1N NaOH.
4. Digest for 30 minutes at room temperature. Make copper reagent.
5. Add 2 mL of copper reagent.
6. Incubate 15 minutes at room temperature.
7. Add 200 μL phenol or folin reagent while vortexing for 15 seconds.
8. Read absorbance on a spectrophotometer at 750 nm.
9. Draw a standard curve by doing a regression of LN protein standards versus LN mean absorbance. The regression line should have an r^2 value of about 0.995. Use the regression equation and the LN mean absorbance of the samples to calculate the LN protein in the sample. Then take the exponent of this value. This tells the amount of protein in the sample (10 μL or 20 μL). Make the appropriate calculations for your use.

Appendix B: Porphyrin Assay

Stock solutions:

ethyl acetate/glacial acetic acid 8:2 (v/v)

1.5 M HCl

Standards: For stock solution (10 μg/mL): 1 mg coproporphyrin III ester is dissolved in 100 mL of 1.5 M HCl. Mix 12.5 mL concentrated HCl with 7.5 mL distilled water and dissolve 1 mg of coproporphyrin III ester in this solution. Leave overnight. Bring volume to 100 mL with distilled water.

Dilution of standards:

1. 200 µL stock solution + 9.8 mL 1.5 M HCl = 0.2 µg/mL
2. 3 mL sol'n 1 + 3.0 mL 1.5 M HCl = 0.1 µg/mL
3. 3 mL sol'n 2 + 3.0 mL 1.5 M HCl = 0.05 µg/mL
4. 3 mL sol'n 3 + 3.0 mL 1.5 M HCl = 0.025 µg/mL
5. 3 mL sol'n 4 + 3.0 mL 1.5 M HCl = 0.0125 µg/mL
6. 3 mL sol'n 5 + 3.0 mL 1.5 M HCl = 0.00625 µg/mL
7. 3 mL sol'n 6 + 3.0 mL 1.5 M HCl = 0.003125 µg/mL
8. 3 mL sol'n 7 + 3.0 mL 1.5 M HCl = 0.0015625 µg/mL

Procedure:

Prepare 3 sets of tubes: 1. 16x100 mm tubes for homogenization. Measure 5 mL of the ethyl acetate/acetic acid mixture into each.
2. 12x75 mm tubes for centrifugation.
3. 12x75 mm tubes for extraction. Measure 4 mL of 1.5 M HCl into each and then withdraw the amount of each sample (step 5 below) from each.

Preparation of the samples:

1. Weigh approximately 20 mg of Harderian gland tissue.
2. Transfer tissue to tube 1 (containing ethyl acetate/glacial acetic acid)
3. Homogenate with polytron.
4. Transfer homogenate to tube 2 and centrifuge at 3000rpm for 30 minutes.
5. Transfer desired amount (suggested 25, 50, or 100 µL) of the supernatant to tube 3 (containing HCl). Vortex.
6. Using fluorescence spectrophotometry, measure fluorescence of the samples and standards at an excitation of 405 nm and an emission of 604 nm.

Calculations:

1. Read the fluorescence of the standards and samples. Plot a calibration curve of emission intensity versus concentration of standards. Using this curve, determine the concentration of the samples in μg/mL.

2. Calculate the porphyrin content of the gland. This can be expressed as μg porphyrins in the whole gland or μg porphyrins /mg of tissue.

A = concentration measured (μg/mL)

B = amount of tissue used in the assay (mg)

C = total weight of the gland (mg)

x = volume of sample used in step 5 above (μL)

Porphyrins (μg/mg tissue)

$$= \frac{\text{A μg porphyrins}}{\text{mL acid}} * \frac{\text{4 mL acid}}{\text{x mL organic}} * 5 \text{ mL organic} \div \text{B mg}$$

$= (A * 20)/(x * B)$ (μg/mg tissue)

$= 800 * A / B$ if 25 μL sampled

$= 400 * A / B$ if 50 μL sampled

$= 200 * A / B$ if 100 μL sampled

Appendix C: One-way Analysis of Variance (ANOVA)

Statistics for ANOVA were calculated according to Khazanie (1986). ANOVA tests variation among three or more groups by comparing it to the variation within the group. The null hypothesis states that all means are equal to each other.

1. Correction term (C.T.): $(\text{grand total})^2$ / number of observations

2. The Between Means Sum of Squares (SSB): $(\text{sample total})^2$ / sample size + ... - C.T.

3. Total Sum of Squares (SST): (each observation)^2 + ... - C.T.

4. Within-Samples Sum of Squares or Error Sum of Squares (SSE): SST-SSB

5. ANOVA Table:

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	Computed ratio
Between	k-1	SSB	SSB/k-1	(SSB/k-1)/(SSE/N-k)
Within	N-k	SSE	SSE/N-k	

6. The F distribution has k-1 and N-k degrees of freedom. From the table of F distribution, $f_{k-1,N-k,0.05}$ is the variance ratio. If the variance ratio exceeds the computed ratio, the null hypothesis is rejected.

Appendix D: Student-Neuman-Kuels Test (SNK)

Statistics were calculated according to Kuehl (1994). SNK compares each treatment mean to each of the other treatment means by a pairwise comparison. The critical values of the test statistic are based upon the distance between the pair of means as they are placed in an ordered array of all treatment means. The null hypothesis states that all means are equal to each other.

1. means are ordered from low to high
2. the harmonic mean (r_h): $[(1/t)(\text{total of } 1/r + ...)]^{-1}$

where r = number of replications per treatment and

t = number of treatments

3. the standard error: $(MSE/r_h)^{1/2}$

where MSE (mean error sum of squares)= SSE/N-k (from ANOVA)

4. The SNK statistic is computed from:

SNK (k,.05)= $q_{.05,k,df}$(standard error) for k=2,3,...t

The critical values of the studentized range statistic and SNK (k,.05) are:

k: 2 3 . . t

$q_{.05,k,df}$ values from table

above values multiplied by standard error

5. Example of SNK table:

		A	D	C	B		
Treatment	Mean	69	71	75	83	k	SNK(k,.05)
A	69		2	6	14	4	7
D	71			4	12	3	6.3
C	75				8	2	5.2
B	83						

The differences between the treatment means run in a diagonal from the upper left to the lower right. If the difference exceeds the critical value, it is significant.

Appendix E: Test of Hypothesis for Coefficient of Linear Correlation

Statistics were calculated according to Khazanie (1986). The null hypothesis states that ρ (the population correlation coefficient) = 0 meaning that there is no linear relationship between the two variables. The test statistic to carry out the test is:

$$r[(n-2)/(1-r^2)]^{1/2}$$

where r = the correlation coefficient.

If the null hypothesis is true, then this statistic has the Student's t distribution with n-2 degrees of freedom. The decision rule is to reject the null hypothesis if the computed value of the test statistic is less than $-t_{n-2,\alpha/2}$ or greater than $t_{n-2,\alpha/2}$

Appendix F:

Sampling Data

	Group	Weight		
		Body (g)	Ovaries (mg)	Uterus (mg)
Females	Int-LD	175 ± 12	50 ± 2	376 ± 16
	Tes-LD	144 ± 4	40 ± 2	551 ± 44 ♦♦
	Int-SD	157 ± 6	40 ± 3	170 ± 25 ♦
	Pnx-SD	167 ± 7	52 ± 4	365 ± 17

	Group	Weight		
		Body (g)	Testes (g)	ASG (mg)
Males	Int-LD	176 ± 4	3.7 ± 0.1	739 ± 74
	Cas-LD	188 ± 10	-	84 ± 7 *
	Int-SD	166 ± 11	0.4 ± 0.1 **	228 ± 41 **
	Cas-SD	179 ± 10	-	83 ± 9 *
	Pnx-SD	168 ± 8	3.5 ± 0.2	652 ± 57

Abbreviations: Int, intact; Tes, testosterone-treated; Pnx, pinealectomized; Cas, castrated; LD, long photoperiod; SD, short photoperiod; ASG, accessory sex glands. Statistics: $P<0.05$ (ANOVA, SNK): ♦, Int-SD vs. all other groups; ♦♦, Tes-LD vs. all other groups; *, Cas-LD or Cas-SD vs. all other groups; **, Int-SD vs. all other groups.